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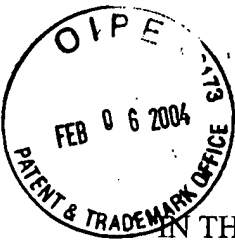
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Lynn Perkes
Serial No. : 09/194,165
Filed : November 20, 1998
Title : DIETARY SUPPLEMENTS CONTAINING NATURAL INGREDIENTS

Art Unit : 1654
Examiner : Patricia Patten

Mail Stop Appeal Brief - Patents

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

BRIEF ON APPEAL

Further to the Notice of Appeal mailed July 30, 2003, Applicant submits the following Brief on Appeal.

(1) Real Party in Interest

The real party in interest is Melaleuca, Inc.

(2) Related Appeals and Interferences

None.

(3) Status of Claims

Claims 1-47 were previously canceled without prejudice.

Claims 48-77 are pending and stand finally rejected.

(4) Status of Amendments

All amendments have been entered.

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(5) Summary of Invention

The invention relates to dietary supplements containing extracts. For example, the present claims recite dietary supplements that contain a grape seed extract, a grape skin extract, and an enzyme, wherein (1) the grape seed extract comprises 3.3 percent or more of the dietary supplement, or (2) the grape seed extract and the grape skin extract combined comprise 32 percent or more of the dietary supplement. The present claims also indicate that the grape seed extract and the grape skin extract contain a flavonoid. In addition, the invention relates to methods for providing a mammal with such dietary supplements. See, e.g., Applicant's specification at page 2, line 26 to page 4, line 5, page 17, lines 16-35, and page 20, line 31 through page 21, line 8.

(6) Issue

Whether claims 48-77 are unpatentable under 35 U.S.C. § 112, first paragraph, for lacking enablement?

(7) Grouping of Claims

Claims 48-77 stand or fall together with respect to the issue of enablement. A different grouping may exist with regard to issues other than this enablement issue.

(8) Argument

Claims 48-77 stand rejected under 35 U.S.C. §112, first paragraph, for lack of enablement on the ground that Applicant's specification does not provide sufficient guidance to enable a person having ordinary skill in the art to make the grape seed extract and grape skin extract recited in the claims. Specifically, the Examiner stated that "there is no teaching in the instant specification what grape seed/skin extract is *actually the invention*," and "[t]he ordinary artisan could *never* determine *what* extract containing *what* flavonoid the claims are referring to, *even after performing undue experimentation* due to lack of information in the [i]nstant specification" (original emphasis).

Applicant respectfully disagrees. Enablement is determined from the viewpoint of “persons of skill in the field of the invention” at the time the patent application was filed. *Ajinomoto Co., Inc. v. Archer DanielsMidland Co.*, 228 F.3d 1338, 56 USPQ2d 1332 (Fed. Cir. 2000). Furthermore, “[a] patent is not a scientific treatise, but a document that presumes a readership skilled in the field of the invention.” *Ajinomoto v. Archer DanielsMidland*, citing *W.L. Gore & Associates, Inc. v. Garlock, Inc.* (721 F.2d 1540, 1556, 220 USPQ 303, 315 (Fed. Cir. 1983)).

The presently claimed invention relates to supplements containing extracts. A person skilled in the art of formulating supplements, reading Applicant's specification at the time Applicant filed, certainly would have been able to make and use the claimed dietary supplements without undue experimentation.

Procedures for making extracts, including grape seed extracts and grape skin extracts, were well known for many years prior to Applicant's filing date. This is particularly true given the prior existence of published references that describe routine methods for making grape seed extracts and/or grape skin extracts. For example, U.S. Patent No. 5,484,594 (the '594 patent) discloses multiple methods for making grape seed extracts. In particular, the '594 patent describes making grape seed extracts using standard extraction, concentration, filtration, and ultrafiltration steps. A skilled artisan would have been able to use these standard methods to prepare grape seed extracts.

In addition, the Revilla *et al.* reference (*J. Agric. Food Chem.*, 46:4592-4597 (1998)) compared grape skin extracts produced using various extraction procedures, including those described in Bourzeix *et al.* (*Bull. O.I.V.*, 59:1171-1254 (1986)), Hebrero *et al.* (*Am. J. Enol. Vitic.*, 39:227-233 (1988)), and Cacho *et al.* (*Am. J. Enol. Vitic.*, 43:244-248 (1992)). See, the paragraph extending from the bottom of the left column to the top of the right column on page 4593 of the Revilla *et al.* reference. The fact that multiple references disclosing such extraction procedures were publicly available prior to Applicant's filing date demonstrates that a person skilled in this art would have been well aware of multiple standard methods for making grape seed extracts and grape skin extracts.

The existence of many standard techniques at the time Applicant filed is further supported by the fact that grape seed extracts and grape skin extracts were commercially available from numerous suppliers. These included, for example, Indena (Milan, Italy), Polyphenolics (Canandaigua, NY), InterHealth (Concord, CA), Tri-K Industries (Fanerson, NJ), Freeman Industries (Tuckahoe, NY), Weinstein Nutritional (Irvine, CA), and Brucia Extracts (Shingle Springs, CA). In fact, Applicant's specification teaches that grape seed extracts and grape skin extracts can be obtained from such suppliers. See, Applicant's specification at page 19, lines 2-5 and 14-16.

Dr. Suk Cho, an employee of Melaleuca, Inc. and a co-inventor on U.S. Application Serial No. 09/800,195, confirmed that grape seed extracts and grape skin extracts were well known and commercially available at the time Applicant filed. See, Paragraph 5 of Dr. Cho's Declaration. Dr. Cho also stated that he would have been able to use standard extraction techniques to make grape seed and grape skin extracts. See, Paragraph 6 of Dr. Cho's Declaration.

Moreover, techniques for preparing formulations containing extracts were routine at the time Applicant's specification was filed. A person skilled in the art would have been able to use routine techniques, such as measuring and mixing techniques, to combine a grape seed extract, a grape skin extract, and an enzyme to make the claimed dietary supplements. Indeed, Dr. Cho stated that he would have been able to measure the amounts of grape seed extract, grape skin extract, and enzyme needed to obtain a dietary supplement recited in present claim 48, and would have been able to mix the measured amounts of grape seed extract, grape skin extract, and enzyme to obtain a dietary supplement recited in present claim 48. See, Paragraph 7 of Dr. Cho's Declaration.

In light of the above, Applicant respectfully requests withdrawal of the rejection of claims 48-77 under 35 U.S.C. § 112, first paragraph.

(9) Appendix

An appendix setting forth the claims on appeal is attached.

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Respectfully submitted,

Date: February 4, 2004

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Appendix of Claims

48. A dietary supplement comprising a grape seed extract, a grape skin extract, and at least one enzyme, wherein said grape seed extract comprises 3.3 percent or more of said supplement by weight, and wherein said grape seed extract and said grape skin extract contain a flavonoid.
49. The supplement of claim 48, wherein said supplement is in the form of a pill, powder, or tablet.
50. The supplement of claim 48, wherein said at least one enzyme comprises a fungal protease, acid stable protease, or bromelain.
51. The supplement of claim 48, wherein said supplement comprises a bilberry extract.
52. The supplement of claim 48, wherein said supplement comprises a ginkgo biloba extract.
53. The supplement of claim 48, wherein said supplement comprises quercetin.
54. The supplement of claim 48, wherein said at least one enzyme comprises about 24 percent or less of said supplement by weight.
55. The supplement of claim 48, wherein said supplement is PROVEXCV™.
56. The supplement of claim 48, wherein said supplement is PROVEXCV2™.
57. The supplement of claim 48, wherein said supplement comprises a ginkgo biloba extract, a bilberry extract, quercetin, a fungal protease, an acid stable protease, and bromelain.

58. A dietary supplement comprising a grape seed extract, a grape skin extract, and at least one enzyme, wherein said grape seed extract and said grape skin extract combined comprise 32 percent or more of said supplement by weight, and wherein said grape seed extract and said grape skin extract contain a flavonoid.
59. The supplement of claim 58, wherein said supplement is in the form of a pill, powder, or tablet.
60. The supplement of claim 58, wherein said at least one enzyme comprises a fungal protease, acid stable protease, or bromelain.
61. The supplement of claim 58, wherein said supplement comprises a bilberry extract.
62. The supplement of claim 58, wherein said supplement comprises a ginkgo biloba extract.
63. The supplement of claim 58, wherein said supplement comprises quercetin.
64. The supplement of claim 58, wherein said at least one enzyme comprises about 24 percent or less of said supplement by weight.
65. The supplement of claim 58, wherein said supplement is PROVEXCV™.
66. The supplement of claim 58, wherein said supplement is PROVEXCV2™.
67. The supplement of claim 58, wherein said supplement comprises a ginkgo biloba extract, a bilberry extract, quercetin, a fungal protease, an acid stable protease, and bromelain.

68. A method for providing a mammal with a dietary supplement, said method comprising administering said supplement to said mammal, wherein said supplement comprises a grape seed extract, a grape skin extract, and at least one enzyme, wherein said grape seed extract and said grape skin extract contain a flavonoid, and wherein:

(a) said grape seed extract comprises 3.3 percent or more of said supplement by weight,
or

(b) said grape seed extract and said grape skin extract combined comprise 32 percent or more of said supplement by weight.

69. The supplement of claim 68, wherein said supplement is in the form of a pill, powder, or tablet.

70. The supplement of claim 68, wherein said at least one enzyme comprises a fungal protease, acid stable protease, or bromelain.

71. The supplement of claim 68, wherein said supplement comprises a bilberry extract.

72. The supplement of claim 68, wherein said supplement comprises a ginkgo biloba extract.

73. The supplement of claim 68, wherein said supplement comprises quercetin.

74. The supplement of claim 68, wherein said at least one enzyme comprises about 24 percent or less of said supplement by weight.

75. The supplement of claim 68, wherein said supplement is PROVEXCV™.

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76. The supplement of claim 68, wherein said supplement is PROVEXCV2™.
77. The supplement of claim 68, wherein said supplement comprises a ginkgo biloba extract, a bilberry extract, quercetin, a fungal protease, an acid stable protease, and bromelain.



Applicants' Copy

Comparison of Several Procedures Used for the Extraction of Anthocyanins from Red Grapes

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Several procedures for the extraction of grape anthocyanins, based on different methods described in the literature, have been assayed. Results have shown that the use of solvents containing up to 1% of 12 N hydrochloric acid for the extraction of grape anthocyanins resulted in the partial hydrolysis of malvidin 3-O-acetylglucoside, leading to important changes in the relative content of anthocyanins in those extracts, despite their higher efficiency in extracting total anthocyanins in some cases. Nevertheless, the use of different neutral solvents resulted in a similar relative content of anthocyanins in the extracts, and some of them could be as efficient in extracting total anthocyanins as other procedures that used an acid solvent for extraction. These data suggest that the use of solvents containing up to 1% of 12 N hydrochloric acid in the extraction of anthocyanins from grapes or from any other plant material containing acetylated anthocyanins should be avoided, especially if the work is related with any type of taxonomic studies.

Keywords: Anthocyanins; acylated anthocyanins; grapes; extraction; HPLC

INTRODUCTION

Anthocyanins are a group of naturally occurring phenolic compounds that are related with the color of several aerial and subterranean organs in many plants (Mazza and Miniatti, 1994). In grapevines (*Vitis vinifera* L.) anthocyanins are accumulated in leaves during senescence and are responsible for the coloration of grape skins in red and rosé cultivars, and in some grape cultivars, named teinturier or redjuice dyer cultivars, these pigments are also accumulated in the grape pulp (Boulton et al., 1995). The accumulation of anthocyanins in grapes begins after the phenological stage known as veraison and is affected by weather conditions, especially light intensity and temperature (Kliwer, 1977), which limit the cultivation of red grape cultivars in regions too cool or too warm during grape maturation. When grapes are harvested, the content of anthocyanins in grapes may be as high as 1000 mg/kg of grapes and even more (Boulton et al., 1995), especially when sugar content in grapes rises to 22%. In red grape cultivars used for wine-making, the amount of anthocyanins present in grapes when harvested may be important in conducting the fermentation and the postfermentation processes in an adequate way to obtain high-quality products. For this reason, several approaches have been taken to understand what changes occur in the content of anthocyanins from veraison to the harvest in several grape cultivars used for wine-making (Piergiovanni and Volonteri, 1983; Darné, 1988; González-San José et al., 1990; Cacho et al., 1992; Fernández-López et al., 1992), showing that the amount of some anthocyanins may decrease in the later stages of the maturation process.

Anthocyanins are unique molecules among plant phenolics because they can be present in plant tissues as different chemical species. At low pH, they are

predominantly present in the form flavylium cation, giving a reddish color in aqueous solutions. At higher pH, the flavylium cation is converted into other species, some of them being uncolored and, under certain conditions, that conversion can be virtually irreversible (Cheminat and Brouillard, 1986). The flavylium cation form is red and stable at a highly acid medium, and this chemical feature of anthocyanins has probably led to a worldwide use of solvents containing mineral or organic acids for the extraction of anthocyanins from plant organs (Ribéreau-Gayon, 1972; Macheix et al., 1990). Nevertheless, Anderson et al. (1970), who identified acetic acid as an acylating agent of anthocyanin pigments in grapes, reported that facile hydrolysis of anthocyanin acetates occurs upon the exposure to trace quantities of mineral acid during the extraction processes, and some authors working on chemotaxonomic studies have reported that the extraction of some acylated anthocyanins under acid conditions may cause their partial or total hydrolysis (Van Sumere et al., 1985; Van Wyck and Winter, 1994). Moreover, Bakker and Timberlake (1985) have shown that artifacts can be obtained by using methanol containing 2% formic acid as the extraction solvent. Despite these reports, several authors have continued to use extraction solvents containing formic acid or hydrochloric acid for studying anthocyanins in grapes (Hebrero et al., 1988; González-San José et al., 1990; Cacho et al., 1992; Fernández-López et al., 1992; Gao and Cahoon, 1995), and some of these methods are also highly time-consuming. Moreover, the use of acid solvents for the extraction of anthocyanins may produce the generation of anthocyanidins from flavanols and proanthocyanidins (Hemingway, 1989), which are present in grape skins to some extent (Bourzeix et al., 1986), and this reaction may result in an overestimation of the total anthocyan content if a colorimetric method is used for this purpose. All of these facts could limit the potential use of those

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Table 1. Some Characteristics of Extraction Procedures Carried out with Skins of Fresh Grapes

| procedure | solvent(s) | vol (mL) | length of extraction (h) | extraction temp (°C) | no. of grapes |
|-----------|--------------------------|---------------|--------------------------|----------------------|---------------|
| 11 | methanol | 60 | 16 | -25 | 100 |
| | 80% methanol | 60 | 4 | 25 | |
| 12 | methanol | 60 | 16 | -25 | 100 |
| | 80% methanol | 60 | 4 | room | |
| | 50% methanol | 60 | 4 | room | |
| | deionized water | 60 | 16 | -25 | |
| | 75% acetone | 60 | 1 | room | |
| 13 | methanol | 60 | 4 | 25 | 100 |
| | methanol | 60 | 12 | 25 | |
| | methanol | 60 | 4 | 25 | |
| 14 | methanol/12 N HCl (99:1) | 60 | 4 | 25 | 100 |
| | methanol/12 N HCl (99:1) | 60 | 12 | 25 | |
| | methanol/12 N HCl (99:1) | 60 | 4 | 25 | |
| | methanol/12 N HCl (99:1) | 60 | 4 | 25 | |
| 15 | methanol/12 N HCl (99:1) | 10 (9 times) | 1 (9 times) | 25 | 50 |
| 16 | methanol | 10 (9 times) | 1 (9 times) | 25 | 50 |
| 17 | methanol/12 N HCl (98:2) | 250 (once) | 24 (once) | 4 | 100 |
| | methanol/12 N HCl (98:2) | 100 | 24 | 4 | |
| | methanol/12 N HCl (98:2) | 100 (4 times) | 24 (4 times) | 4 | |

procedures for routine analysis of anthocyanins in grapevine production.

The aim of our research has been to develop a rapid method, avoiding the use of acid extractions solvents, for the extraction of anthocyanins from grapes that could be used as a routine analysis for enhancing grapevine production. To achieve this purpose, several extraction procedures with different type of solvents (acid or neutral) have been compared using the same set of grapes, and their efficiency in the extraction of anthocyanins has been estimated by colorimetric and chromatographic methods.

EXPERIMENTAL PROCEDURES

Grape Samples. One hundred clusters (~10 kg) of Cabernet Sauvignon grapes were collected in October 1996 from 100 different vines, trained in Guyot double cordon, in a vineyard located in Escalona (province of Toledo, Spain), which has been surveyed by our group for several years. The clusters were collected in plastic bags and were transported to the laboratory at 4 °C. Once there, the grapes were separated from the clusters. Thirty sets of 100 grapes and six sets of 50 grapes were randomly selected and weighed.

Extraction Procedures. Immediately after grape sets were randomly selected and weighed, all of them were processed by carrying out several extraction procedures consisting of two or more sequential steps, the characteristics of which are displayed in Tables 1 and 2. Each extraction procedure has been identified by a two-digit number; the first digit refers to the type of material to be extracted (skins of fresh grapes or entire fresh grapes, designated 1 and 2, respectively), and the second digit refers to the characteristics of the extraction process. Thus, the procedures 11 and 16 involved the extraction of fresh grape skins; and procedures 13 and 23 were carried out under the same conditions, but using skins of fresh grapes and entire fresh grapes, respectively. Some of the procedures used are based on methods for the extraction of phenolic compounds described in the literature. Procedures 12 and 22 are based on the method developed

Table 2. Some Characteristics of Extraction Procedures Carried out with Entire Fresh Grapes

| procedure | solvent(s) | vol (mL) | length of extraction (h) | extraction temp (°C) | no. of grapes |
|-----------|--------------------------|---------------|--------------------------|----------------------|---------------|
| 21 | methanol | 60 | 16 | -25 | 100 |
| | 80% methanol | 60 | 4 | 25 | |
| 22 | methanol | 60 | 16 | -25 | 100 |
| | 80% methanol | 60 | 4 | room | |
| | 50% methanol | 60 | 4 | room | |
| | deionized water | 60 | 16 | -25 | |
| | 75% acetone | 60 | 1 | room | |
| 23 | methanol | 60 | 4 | 25 | 100 |
| | methanol | 60 | 12 | 25 | |
| | methanol | 60 | 4 | 25 | |
| 24 | methanol/12 N HCl (99:1) | 60 | 4 | 25 | 100 |
| | methanol/12 N HCl (99:1) | 60 | 12 | 25 | |
| | methanol/12 N HCl (99:1) | 60 | 4 | 25 | |
| | methanol/12 N HCl (99:1) | 60 | 4 | 25 | |
| 27 | methanol/12 N HCl (98:2) | 250 (once) | 24 (once) | 4 | 100 |
| | methanol/12 N HCl (98:2) | 100 | 24 | 4 | |
| | methanol/12 N HCl (98:2) | 100 (4 times) | 24 (4 times) | 4 | |

by Bourzeix et al. (1986) for extracting phenolic compounds from grape skins and grape seeds; procedures 17 and 27 are based on the method described by Hebrero et al. (1988) for the anthocyanin extraction from grape skins, and procedure 15 is based on the method proposed by Cacho et al. (1992) for extracting anthocyanins from grape skins. As can be noted, each procedure involves two or more successive extractions, and all of them include, as a first step, the immersion of the sample in pure methanol (which contains an adequate proportion of 12 N hydrochloric acid if necessary), followed by grinding in a Kinematica PCU-2 blender for 1 min. When each extraction step was finished, the liquid extract was separated by centrifugation at 3500 rpm and 15 °C for 20 min in a Sorvall RC-5B refrigerated centrifuge, and the residue was submitted to extraction again. For each sample, all of the liquid extracts were combined, and their volume was raised to between 200 and 650 mL with methanol. The extracts were then stored at 4 °C prior to their analysis. Each extraction procedure was carried out in triplicate.

Colorimetric Analysis. Total phenolics were measured in the extracts according to the Folin-Ciocalteu method, following the procedure proposed by Singleton and Rossi (1965), and were expressed as gallic acid equivalents. Total anthocyanins, expressed as malvidin 3-O-glucoside equivalents, were determined using the procedure proposed by Niketic-Aleksic and Hrazdina (1972). All of these measurements were carried out in a Turner 690 UV-vis spectrophotometer.

HPLC Analysis. Several anthocyanins present in the extracts were separated and determined by HPLC. Routine analyses were performed in a chromatograph that consisted of Waters M-510 and M-501 solvent delivery systems, a Waters 680 gradient controller, a Rheodyne 7725 injection valve furnished with a 20 µL loop, a Linear UVIS 200 variable wavelength UV-vis detector set at 520 nm with a sensitivity of 0.01 AUFS, and a Spectra-Physics SP-4290 integrator. In some cases, detection was carried out with a Waters 996 photodiode array detector, and data were acquired and processed in a Waters Millennium workstation, vs 2.15.01, the spectra being registered each second between 250 and 600 nm, with a bandwidth of 1.2 nm. The chromatographic separation was performed on a Waters Nova-Pak C18 cartridge, 150 mm × 3.9 mm, using a Waters Sentry Nova-Pak C18 guard column, 20 mm × 3.9 mm, both thermostated in a water bath at 32 °C. The mobile phase was a linear gradient of water/acetonitrile (60:40) adjusted at pH 1.2 with perchloric acid (solvent B) in deionized distilled water adjusted at pH 1.2 with perchloric acid (solvent A), at a flow rate of 1.5 mL/min, as it is shown in

Table 3. Linear Gradient Used for the Separation of Anthocyanins by HPLC

| time (min) | solvent A (%) | solvent B (%) | time (min) | solvent A (%) | solvent B (%) |
|------------|---------------|---------------|------------|---------------|---------------|
| 0 | 90 | 10 | 54 | 67 | 33 |
| 5 | 90 | 10 | 55 | 0 | 100 |
| 25 | 80 | 20 | 58 | 0 | 100 |
| 45 | 70 | 30 | 59 | 90 | 10 |

Table 4. Extraction of Total Anthocyanins and Total Phenolics from Skins of Fresh Grapes^a

| extraction procedure | total anthocyanins (mg/100 g of grapes) | total phenolics (mg/100 g of grapes) |
|----------------------|---|--------------------------------------|
| 11 | 79.7 ± 3.7a | 182 ± 10b |
| 12 | 82.8 ± 2.1ab | 248 ± 2c |
| 13 | 88.3 ± 3.1ab | 173 ± 8b |
| 14 | 94.0 ± 7.4b | 243 ± 14c |
| 15 | 91.3 ± 5.6ab | 194 ± 15b |
| 16 | 86.6 ± 4.4ab | 111 ± 3a |
| 17 | 113.9 ± 1.8c | 267 ± 10c |

^a Data are mean values for three replications. Values in the same column followed by a common letter are not significantly different ($p < 0.01$).

Table 3. Under these conditions, which are quite close to those described by Bakker and Timberlake (1985), acylated anthocyanins are reasonably stable. The different anthocyanins that have been analyzed were identified on the basis of their retention times and UV-vis spectra by comparison with standards isolated from grapes, as indicated elsewhere. The quantitation of the different substances was carried out in the chromatograms registered at 520 nm by an external standard procedure, using a series of solutions of malvidin 3-*O*-glucoside isolated from grapes. Results are expressed as malvidin 3-*O*-glucoside equivalents.

Statistical Analysis. Statistical analyses of multiple treatment effects were conducted by using one-way analysis of variance with comparison of means made by Duncan's multiple-range test at the 1% level of significance (Duncan, 1957).

RESULTS AND DISCUSSION

Total Phenolics and Total Anthocyanins. Table 4 displays the data corresponding to the levels of total phenolics and total anthocyanins in the different extraction procedures carried out with skins of fresh grapes. As can be noted, the extraction of total phenolics clearly differs in some cases, and two factors seem to play an important role: the total time employed in the extraction and the use of solvents containing hydrochloric acid. Thus, procedure 16, which involves the use of methanol for 9 h, led to the lowest level of extraction of phenolics among the seven procedures used ($p < 0.01$). On the other hand, procedure 17, which used methanol containing 1% of 12 N HCl for a very long time (120 h), led to the highest extraction of phenolics among the seven procedures assayed, but procedures 12 and 14 were as efficient in extracting phenolics as procedure 17 ($p < 0.01$), despite the use of neutral solvents for extraction for the long time (procedure 12) or the relatively short time (20 h) skins were in contact with an acid extraction solvent (procedure 14). The dramatic effect of acidity on the extraction of phenolics is clearly shown by the results corresponding to procedures 15 and 16, which differ only by the presence of hydrochloric acid in the extraction solvent used in procedure 15, and it is a likely consequence of the association of phenolic compounds with cell wall polymers, although the presence of an acid in solvent should increase the rate of extraction of those substances.

Table 5. Extraction of Total Anthocyanins and Total Phenolics from Entire Fresh Grapes^a

| extraction procedure | total anthocyanins (mg/100 g of grapes) | total phenolics (mg/100 g of grapes) |
|----------------------|---|--------------------------------------|
| 21 | 83.5 ± 2.1b | 311 ± 33a |
| 22 | 70.8 ± 1.9a | 430 ± 36b |
| 23 | 86.1 ± 5.5b | 357 ± 7a |
| 24 | 81.1 ± 2.6ab | 510 ± 26c |
| 27 | 110.9 ± 6.4c | 561 ± 7c |

^a Data are mean values for three replications. Values in the same column followed by a common letter are not significantly different ($p < 0.01$).

The effects of different factors in the extraction of anthocyanins in experiments carried out with skins of fresh grapes were less evident than they were for total phenolics, but the acidity of solvents used for the extraction and the length of the extraction may play a relevant role in the extraction of those substances. Thus, procedure 17, which used methanol containing 2% of 12 N HCl as solvent, led to a significantly higher extraction of anthocyanins than any other procedure ($p < 0.01$), and procedure 14, which used methanol containing 1% of 12 N HCl as solvent for 20 h, was more efficient ($p < 0.01$) than procedure 11, which used neutral solvents for 20 h. It is very probable that the higher efficiency of procedure 17 was a consequence of the much longer extraction time associated with it compared to others, but it could be hypothesized that the higher efficiency of acid solvents for extracting anthocyanins could partially be explained because flavanols and proanthocyanidins, which are present in grape skins to some extent (Bourzeix et al., 1986), generate anthocyanidins in acid media (Hemingway, 1989). Nevertheless, this hypothesis needs further work to be confirmed.

Table 5 shows the data corresponding to the levels of total phenolics and total anthocyanins in the different experiments carried out with entire fresh grapes. Results obtained for total phenolics were higher than those obtained for assays carried out with skins of fresh grapes (Table 3), as grape seeds contain a very large amount of phenolics (Bourzeix et al., 1986), but the effects of two factors (acidity of solvents and total extraction time) on the extraction of total phenolics from entire fresh grapes were quite similar to those observed in the extraction assays carried out with skins of fresh grapes. It can be seen that the levels of total phenolics were significantly higher ($p < 0.01$) in the extracts corresponding to experiments carried out with acid solvents (procedures 24 and 27) than in the extracts obtained with neutral solvents (procedures 21–23), and this effect should be explained as mentioned above. However, the effects of different factors on the extraction of anthocyanins were less evident than they were for total phenolics: procedure 27, which involved the use of an acid solvent for 120 h, apparently led to the highest extraction ($p < 0.01$) among the five procedures used, but the efficiency of procedures 21, 23, and 24, which lasted 20 h, was similar for extracting anthocyanins ($p < 0.01$), despite the use of neutral solvents (procedures 21 and 23) or of a solvent containing hydrochloric acid (procedure 24). Finally, procedure 22 led to the extraction of a very low amount of anthocyanins. As has been mentioned above, the apparently higher efficiency of procedure 27 for extracting anthocyanins may be a consequence of the much longer extraction time associated with it compared to the others, but the possible

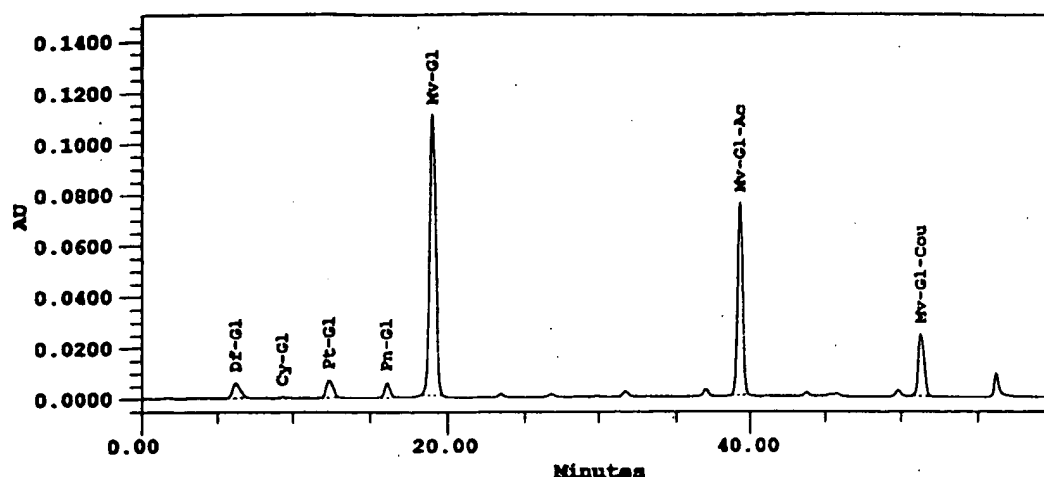


Figure 1. Chromatogram registered at 520 nm corresponding to an extract obtained from skins of fresh grapes, following procedure 11. For key to substances, see text.

Table 6. Extraction of Several Anthocyanins from Skins of Fresh Grapes^a

| | extraction procedure | | | | | | |
|-----------|----------------------|-------------|--------------|--------------|-------------|--------------|-------------|
| | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| Df-Gl | 4.2 ± 0.5a | 4.4 ± 0.4a | 4.9 ± 0.5ab | 5.6 ± 0.5b | 5.8 ± 0.1b | 4.3 ± 0.5a | 5.6 ± 0.5b |
| Cy-Gl | 0.3 ± 0.1a | 0.3 ± 0.1ab | 0.4 ± 0.1abc | 0.5 ± 0.0c | 0.5 ± 0.1bc | 0.4 ± 0.1abc | 0.5 ± 0.1c |
| Pt-Gl | 3.2 ± 0.3a | 3.4 ± 0.2ab | 3.6 ± 0.3abc | 4.4 ± 0.4bcd | 4.6 ± 0.2cd | 3.4 ± 0.4ab | 5.0 ± 0.4d |
| Pn-Gl | 2.2 ± 0.2a | 2.3 ± 0.1a | 2.4 ± 0.3a | 3.2 ± 0.1b | 3.4 ± 0.2bc | 2.3 ± 0.3a | 3.8 ± 0.2c |
| Mv-Gl | 37.9 ± 2.7a | 43.3 ± 2.0a | 42.0 ± 1.2a | 59.2 ± 4.7b | 65.8 ± 2.0b | 41.2 ± 3.8a | 77.8 ± 2.2c |
| Mv-Gl-Ac | 23.1 ± 2.2d | 25.5 ± 0.3e | 25.7 ± 1.1e | 10.2 ± 0.6c | 3.9 ± 0.1b | 25.3 ± 2.1de | 0.5 ± 0.1a |
| Mv-Gl-Cou | 8.1 ± 0.7ab | 9.1 ± 0.2bc | 8.6 ± 0.4ab | 10.1 ± 0.8cd | 10.6 ± 0.1d | 7.4 ± 0.8a | 10.4 ± 0.3d |

^a Results are expressed as mg of Mv-Gl equivalents/100 g of grapes. Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

Table 7. Extraction of Several Anthocyanins from Entire Fresh Grapes^a

| | extraction procedure | | | | |
|-----------|----------------------|-------------|-------------|-------------|-------------|
| | 21 | 22 | 23 | 24 | 27 |
| Df-Gl | 4.2 ± 0.3a | 3.7 ± 0.4a | 3.8 ± 0.4a | 5.1 ± 0.3b | 5.5 ± 0.4b |
| Cy-Gl | 0.3 ± 0.0ab | 0.3 ± 0.1a | 0.3 ± 0.1ab | 0.4 ± 0.1ab | 0.4 ± 0.1b |
| Pt-Gl | 3.2 ± 0.2a | 2.9 ± 0.1a | 3.0 ± 0.3a | 3.7 ± 0.2a | 4.8 ± 0.4b |
| Pn-Gl | 2.1 ± 0.1a | 2.1 ± 0.1a | 2.2 ± 0.1a | 2.5 ± 0.1b | 3.9 ± 0.2c |
| Mv-Gl | 39.6 ± 1.7a | 34.9 ± 1.1a | 39.5 ± 1.7a | 45.3 ± 1.4b | 73.5 ± 3.2c |
| Mv-Gl-Ac | 23.8 ± 1.9cd | 21.8 ± 0.5c | 24.9 ± 0.6d | 13.1 ± 1.2b | 0.3 ± 0.2a |
| Mv-Gl-Cou | 8.6 ± 0.5b | 7.4 ± 0.5a | 8.8 ± 0.3b | 9.2 ± 0.4b | 10.9 ± 0.3c |

^a Results are expressed as mg of Mv-Gl equivalents/100 g of grapes. Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

formation of anthocyanidins from flavanols and proanthocyanidins extracted from grape skins and seeds in an acid media should be considered to a certain extent (Hemingway, 1989).

Extraction of Individual Anthocyanins. Figure 1 shows a chromatogram corresponding to an extract from skins of fresh grapes obtained by procedure 11. Seven different peaks were quantitated and were assigned to delphinidin 3-*O*-glucoside (Df-Gl), cyanidin 3-*O*-glucoside (Cy-Gl), petunidin 3-*O*-glucoside (Pt-Gl), peonidin 3-*O*-glucoside (Pn-Gl), malvidin 3-*O*-glucoside (Mv-Gl), malvidin 3-*O*-acetylglucoside (Mv-Gl-Ac), and malvidin 3-*O*-*p*-coumarylglucoside (Mv-Gl-Cou) on the basis of their chromatographic and spectral characteristics, as has been previously reported (Wulf and Nagel, 1978; Bakker and Timberlake, 1985; Hebrero et al., 1988; Baldi et al., 1995).

Tables 6 and 7 display the data corresponding to the amount of seven anthocyanins determined by HPLC in the extracts obtained using the different procedures assayed for skins of fresh grapes and for entire fresh

grapes, respectively. It can be noticed that the use of solvents which contained hydrochloric acid for extracting anthocyanins apparently led to a higher extraction of nonacylated anthocyanins, and especially malvidin 3-*O*-glucoside, which is the major anthocyanin in Cabernet Sauvignon grapes (Wulf and Nagel, 1978; Darné, 1988). The use of acid solvents (procedures 14, 15, and 17 in the case of experiments with skins of fresh grapes, and procedures 24 and 27 for experiments with entire fresh grapes) led to extracts that contained more malvidin 3-*O*-glucoside than those extracts obtained by procedures that used neutral solvents ($p < 0.01$). The effect was especially intense when methanol containing 2% of 12 N HCl was used as solvent. The other four nonacylated anthocyanins (3-*O*-glucosides of delphinidin, cyanidin, petunidin, and peonidin) behaved similarly, and procedures 17 and 27 led to the highest amount of these molecules in the extracts.

The two acylated anthocyanins that have been analyzed (malvidin 3-*O*-acetylglucose and malvidin 3-*O*-*p*-coumarylglucose) behaved in different ways. Appar-

Table 8. Area Percentages of Seven Anthocyanins Determined by HPLC in Extracts Obtained from Skins of Fresh Grapes^a

| | extraction procedure | | | | | | |
|-----------|----------------------|--------------|--------------|--------------|-------------|-------------|-------------|
| | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| Df-Gl | 5.3 ± 0.3a | 5.0 ± 0.3a | 5.6 ± 0.3abc | 6.0 ± 0.1bc | 6.1 ± 0.0c | 5.1 ± 0.3a | 5.4 ± 0.4ab |
| Cy-Gl | 0.3 ± 0.1a | 0.3 ± 0.1a | 0.5 ± 0.1ab | 0.5 ± 0.0b | 0.5 ± 0.1ab | 0.4 ± 0.0ab | 0.5 ± 0.1ab |
| Pt-Gl | 4.1 ± 0.1a | 3.9 ± 0.1a | 4.1 ± 0.3a | 4.7 ± 0.1b | 4.8 ± 0.0b | 4.1 ± 0.1a | 4.9 ± 0.2b |
| Pn-Gl | 2.8 ± 0.1a | 2.6 ± 0.1a | 2.7 ± 0.3a | 3.5 ± 0.2b | 3.6 ± 0.1b | 2.8 ± 0.1a | 3.7 ± 0.1b |
| Mv-Gl | 48.1 ± 0.6a | 49.0 ± 0.9a | 48.0 ± 0.1a | 63.6 ± 0.3b | 69.7 ± 0.4c | 48.9 ± 0.0a | 75.1 ± 0.7d |
| Mv-Gl-Ac | 29.2 ± 0.5d | 28.9 ± 1.1d | 29.4 ± 0.7d | 10.9 ± 0.2c | 4.1 ± 0.1b | 30.0 ± 0.0d | 0.5 ± 0.1a |
| Mv-Gl-Cou | 10.3 ± 0.2bc | 10.3 ± 0.2bc | 9.8 ± 0.1b | 10.8 ± 0.0cd | 11.2 ± 0.4d | 8.7 ± 0.2a | 10.1 ± 1.2b |

^a Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

Table 9. Area Percentages of Seven Anthocyanins Determined by HPLC in Extracts Obtained from Entire Fresh Grapes^a

| | extraction procedure | | | | |
|-----------|----------------------|-------------|--------------|-------------|--------------|
| | 21 | 22 | 23 | 24 | 27 |
| Df-Gl | 5.1 ± 0.1ab | 5.0 ± 0.6ab | 4.6 ± 0.3a | 6.4 ± 0.2c | 5.6 ± 0.2b |
| Cy-Gl | 0.4 ± 0.0a | 0.4 ± 0.1a | 0.4 ± 0.1a | 0.5 ± 0.1a | 0.4 ± 0.0a |
| Pt-Gl | 4.0 ± 0.2a | 4.0 ± 0.1a | 3.7 ± 0.3a | 4.7 ± 0.2b | 4.9 ± 0.2b |
| Pn-Gl | 2.6 ± 0.2a | 2.8 ± 0.1ab | 2.7 ± 0.1a | 3.1 ± 0.1b | 3.9 ± 0.1c |
| Mv-Gl | 48.4 ± 0.8a | 47.9 ± 0.8a | 47.9 ± 0.3a | 57.2 ± 1.1b | 74.0 ± 0.3c |
| Mv-Gl-Ac | 29.0 ± 0.8c | 29.9 ± 0.2c | 30.2 ± 0.4c | 16.5 ± 0.9b | 0.3 ± 0.2a |
| Mv-Gl-Cou | 10.5 ± 0.5ab | 10.1 ± 0.4a | 10.6 ± 0.5ab | 11.6 ± 0.2b | 11.0 ± 0.2ab |

^a Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

ently, the extraction of malvidin 3-*O*-*p*-coumarylglucose followed the same patterns as the extraction of nonacylated anthocyanins. Certainly, the amount of malvidin 3-*O*-*p*-coumarylglucose in extracts obtained from skins of fresh grapes (Table 5) was higher in procedures that involved the use of hydrochloric acid (procedures 14, 15, and 17) than in the others, but differences were not so important as those observed for malvidin 3-*O*-glucoside, and extractions carried out by procedures 12 and 14 were not different ($p < 0.01$). A similar effect was observed for extracts obtained from entire fresh grapes: procedure 27, which involved the use of an acid solvent, led to a more intense extraction of malvidin 3-*O*-*p*-coumarylglucose than procedures 21–23, which used neutral solvents, but the extraction carried out by procedure 24, which involved the use of an acid solvent, was not different ($p < 0.01$) from the extractions carried out by procedures 21 and 22, which used neutral solvents. Consequently, it would be possible to hypothesize that the factors that affect the extraction of malvidin 3-*O*-*p*-coumarylglucose from grapes may be qualitatively different from those that influence the extraction of malvidin 3-*O*-glucoside, except if the high amount of this latter molecule in extracts obtained with acid solvents was the consequence of the hydrolysis of any other acylated derivative. On the other hand, procedures that involved the use of acidic solvents (procedures 14, 15, 17, 24, and 27) led to the extraction of a lower amount of malvidin 3-*O*-acetylglucose ($p < 0.01$) than procedures that used neutral solvents. The effect of using methanol containing 2% of 12 N HCl as solvent was especially dramatic, and the extracts obtained with this solvent contained a very low amount of malvidin 3-*O*-acetylglucose. The effect of other factors on the extraction of nonacylated anthocyanins and their acetyl derivatives, such as total extraction time, extraction temperature, or the number of successive extractions, seem to be less important than the use of acidic solvents for their extraction.

The results obtained suggest that the presence of hydrochloric acid in the extraction solvent originates the partial hydrolysis of acetylated anthocyanins, as it was reported previously by Anderson et al. (1970), Van Sumere et al. (1985), and Van Wyk and Winter (1994). Data on the relative content of the seven anthocyanins analyzed by HPLC in the extracts obtained in the different experiments, expressed as area percentages and summarized in Tables 8 and 9, would make this question clear. The relative contents of malvidin 3-*O*-glucoside were quite similar for all of the extracts obtained with neutral solvents (procedures 11–13, 16, and 21–23), ranging from 47.9% (procedure 22) to 49.0% (procedure 12), and, in the same way, the relative contents of malvidin 3-*O*-acetylglucose were quite similar for all of the extractions carried out with neutral solvents, ranging from 28.9% (procedure 12) to 30.2% (procedure 23). The use of acid solvents increased the relative content of malvidin 3-*O*-glucoside in the extracts ($p < 0.01$), which may be as high as 75.1% (procedure 17), and consequently reduced the relative content of malvidin 3-*O*-acetylglucose ($p < 0.01$), which may be as low as 0.3% (procedure 27).

Furthermore, the relative contents of the other four nonacylated anthocyanins (the 3-*O*-glucosides of delphinidin, cyanidin, petunidin, and peonidin) were higher in the extracts obtained by procedures that involved the use of acid solvents than in those obtained when neutral solvents were used, probably as a consequence of the hydrolysis of their acetylated derivatives, because the apparent extraction of those nonacylated anthocyanins was more intense when acid solvents were used (Tables 6 and 7). Unfortunately, it was not possible to evaluate the amount of the acetyl derivatives of those anthocyanins present in the extracts.

These data point out that the use of solvents containing up to 1% of 12 N hydrochloric acid produces the partial hydrolysis of some acetylated anthocyanins during extraction. As a consequence, the use of experi-

mental protocols for extracting anthocyanins using solvents that contained up to 1% of 12 N hydrochloric acid from grapes or from any other plant material containing acetylated anthocyanins should be avoided, especially if the work is related with any type of taxonomic studies. Any extraction protocol that uses other mineral and organic acids, and even solvents containing hydrochloric acid at lower concentrations than those used in our experiments, should be tested against an extraction procedure that involves the use of neutral solvents, to avoid partial hydrolysis of acetylated anthocyanins or even the formation of laboratory artifacts, as those mentioned by Bakker and Timberlake (1985) when formic acid is used for the extraction of anthocyanins. The relatively high amount of malvidin 3-O-acetylglucoside present in Cabernet Sauvignon grapes makes them good material for testing extraction solvents intended to be used for anthocyanin analysis.

Some of the procedures used, and especially procedure 23, could give information of interest about the content of anthocyanins in grapes shortly after sampling (~24 h) and even complementary information about the global phenolic status of grapes. These data could be useful for the better conducting of red wine fermentation, as anthocyanins and other phenolic compounds play a very relevant role in the characteristics of red wines.

LITERATURE CITED

- Anderson, D. W.; Gueffroy, D. E.; Webb, A. D.; Kepner, R. E. Identification of acetic acid as an acylating agent of anthocyanin pigment in grapes. *Phytochemistry* 1970, 9, 1579–1583.
- Bakker, J.; Timberlake, C. F. The distribution of anthocyanins in grape skin extracts of Port wine cultivars as determined by high performance liquid chromatography. *J. Sci. Food Agric.* 1985, 36, 1315–1324.
- Baldi A.; Romani, A.; Mulinacci, N.; Vincieri, F. F.; Casetta, B. HPLC/MS application to anthocyanins of *Vitis vinifera* L. *J. Agric. Food Chem.* 1995, 43, 2104–2109.
- Boulton, R. B.; Singleton, V. L.; Bisson, L. F.; Kunkee, R. E. *Principles and Practices of Winemaking*; Chapman & Hall: New York, 1995.
- Bourzeix, M.; Weyland, D.; Heredia, N. Étude des catéchuins et des procyanidols de la grappe de raisin, du vin, et d'autres dérivés de la vigne (A study of catechins and procyanidins of grape clusters, the wine, and other byproducts of the vine). *Bull. O.I.V.* 1986, 59, 1171–1254.
- Cacho, J.; Fernández, P.; Ferreira, V.; Castell, J. E. Evolution of five anthocyanidin-3-glucosides in the skin of Tempranillo, Moristel, and Garnacha grape varieties and influence of climatological variables. *Am. J. Enol. Vitic.* 1992, 43, 244–248.
- Cheminat, A.; Brouillard, R. PMR investigation of 3-O-(β -D-glucosyl)-malvidin structural transformations in aqueous solutions. *Tetrahedron Lett.* 1986, 27, 4457–4460.
- Darné, G. Evolution des différentes anthocyanes des pellicules de Cabernet Sauvignon au cours du développement des baies (Evolution of different anthocyanins in the skins of Cabernet Sauvignon during grape development). *Connaiss. Vigne Vin* 1988, 22, 225–236.
- Duncan, D. B. Multiple range tests for correlated and heteroscedastic means. *Biometrics* 1957, 13, 164–176.
- Fernández-López, J. A.; Hidalgo, V.; Almela, L.; López-Roca, J. M. Quantitative changes in anthocyanin pigments of *Vitis vinifera* cv. Monastrell during maturation. *J. Sci. Food Agric.* 1992, 58, 153–155.
- Gao, Y.; Cahoon, G. A. High performance liquid chromatographic analysis of anthocyanins in the red seedless table grape Reliance. *Am. J. Enol. Vitic.* 1995, 46, 339–345.
- González-San José, M. L.; Barrón, L. J. R.; Diez, C. Evolution of anthocyanins during maturation of Tempranillo grape variety (*Vitis vinifera*) using polynomial regression models. *J. Sci. Food Agric.* 1990, 51, 337–343.
- Hebrero, E.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. High performance liquid chromatography-diode array spectroscopy identification of anthocyanins of *Vitis vinifera* variety Tempranillo. *Am. J. Enol. Vitic.* 1988, 39, 227–233.
- Hemingway, R. W. Reactions at the interflavonoid bond of proanthocyanins. In *Chemistry and Significance of Condensed Tannins*; Hemingway, R. H., Karchesy, J. J., Eds.; Plenum Press: New York, 1989.
- Kliewer, W. M. Influence of temperature, solar radiation and nitrogen on coloration and composition of Emperor grapes. *Am. J. Enol. Vitic.* 1977, 28, 96–103.
- Macheix, J. J.; Fleuriet, A.; Billot, J. *Fruit Phenolics*; CRC Press: Boca Raton, FL, 1990.
- Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables and Grains*; CRC Press: Boca Raton, FL, 1993.
- Niketic-Aleksic, G. K.; Hrazdina, G. Quantitative analysis of the anthocyanin content in grape juices and wines. *Lebensm. Wiss. Technol.* 1972, 5, 163–165.
- Piergovanni, L.; Volonterio, G. Studio della frazione antocianica delle uve. Nota II. Variazione di composizione durante la maturazione (A study of the anthocyanins fraction of grapes. II. Changes in its composition during ripening). *Tecnol. Aliment. Imbottigliamento* 1983, 6, 22–28.
- Ribéreau-Gayon, P. *Plant Phenolics*; Oliver and Boyd: Edinburgh, 1972.
- Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent. *Am. J. Enol. Vitic.* 1965, 16, 144–158.
- Van Sumere, C. F.; Vande Castele, K.; De Loose, R.; Heursel, J. Reversed phase-HPLC analysis of flavonoids and the biochemical identification of cultivars of evergreen Azalea. In *Annual Proceedings of the Phytochemical Society of Europe*; Van Sumere, C. F., Lea, P. J., Eds.; Clarendon Press: Oxford, U.K., 1985; Vol. 25.
- Van Wyk, B. E.; Winter, P. J. D. Chemotaxonomic value of anthocyanins in *Podalyria* and *Virgilia* (Tribe Podalyrieae: Fabaceae). *Biochem. Syst. Ecol.* 1994, 22, 813–818.
- Wulf, L. W.; Nagel, C. W. High-pressure liquid chromatographic separation of anthocyanins of *Vitis vinifera*. *Am. J. Enol. Vitic.* 1976, 29, 42–49.

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JF9804692



Attorney Docket No. 09143-005001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Lynn Perkes
Serial No. : 09/194,165
Filed : November 20, 1998
Title : DIETARY SUPPLEMENTS CONTAINING NATURAL INGREDIENTS

Art Unit : 1614
Examiner : Patricia Patten

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132 OF SUK CHO

I, Suk Cho, declare as follows:

1. I am a citizen of the United States and presently live at 2610 West Charlotte Drive, Idaho Falls, Idaho, 83402.
2. I am presently employed by Melaleuca Inc., and have been so employed since 1998.
3. I received a Ph.D. in organic chemistry from Emory University, Atlanta, Georgia.
4. I have read the above-reference patent application, pending claims, and the Examiner's Office Actions mailed August 13, 2002 and May 1, 2003, including the sections where the Examiner contends that the skilled artisan would not be able to ascertain how to make grape seed extracts and grape skin extracts upon reading the patent application.
5. Grape seed extracts containing flavonoids and grape skin extracts containing flavonoids were well known as of 1997. In fact, many companies sold grape seed and grape skin extracts containing flavonoids as of 1997.
6. As a chemist, I would have been able to use standard extraction techniques to make grape seed and grape skin extracts containing flavonoids as of 1997. For example,

Attorney Docket No. 09143-005001

I would have been able to follow standard extraction techniques, such as those described in the references discussed in Applicant's Response filed February 12, 2003 (e.g., U.S. Patent No. 5,484,594), to obtain various grape seed and grape skin extracts containing flavonoids. In general, making flavonoid-containing extracts from grape seeds and grape skins involves using routine, well known procedures.

7. Following the teachings in the above-referenced patent application, I would have been able to make dietary supplements containing grape seed and grape skin extracts containing flavonoids as of 1997. For example, I would have been able to measure the amounts of grape seed extract, grape skin extract, and enzyme needed to obtain a dietary supplement recited in present claim 48. In addition, I would have been able to mix the measured amounts of grape seed extract, grape skin extract, and enzyme to obtain a dietary supplement recited in present claim 48. Measuring and mixing amounts of grape seed extract, grape skin extract, and enzyme involves standard laboratory techniques.
8. Following the teachings in the above-referenced patent application, I would have been able to evaluate dietary supplements for the ability to inhibit platelet aggregation. For example, I would have been able to follow the *ex vivo* procedures described in Example 1 of the above-referenced patent application to determine whether a dietary supplement inhibited platelet aggregation. The techniques used to assess platelet aggregation are standard techniques that can be performed by a trained laboratory technician.
9. Likewise, following the teachings in the above-referenced patent application, I would have been able to evaluate dietary supplements for the ability to protect LDL cholesterol from oxidation. For example, I would have been able to follow the procedures described in Example 2 of the above-referenced patent application to determine whether a dietary supplement protected LDL cholesterol from oxidation. The techniques used to assess oxidation of LDL cholesterol are standard techniques that can be performed by a trained laboratory technician.

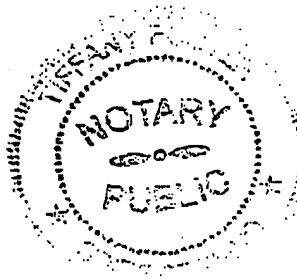
Attorney Docket No. 09143-005001

10. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant patent application or any patent issuing thereon.

Dated: July 1, 2003Suk Cho
Suk Cho

STATE OF Idaho)
) ss.
COUNTY OF Bonniville)

Before me this 1 day of July, 2003, personally appeared Suk Cho known to me to be the person whose name is subscribed to the foregoing Declaration, and acknowledged that he executed the same as his free act and deed for the purposes therein contained.

Tiffany Foster
Notary Public

60148941.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Lynn Perkes
Serial No. : 09/194,165
Filed : November 20, 1998
Title : DIETARY SUPPLEMENTS CONTAINING NATURAL INGREDIENTS

Art Unit : 1654
Examiner : Patricia Patten

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

BRIEF ON APPEAL

Further to the Notice of Appeal mailed July 30, 2003, Applicant submits the following
Brief on Appeal.

(1) Real Party in Interest

The real party in interest is Melaleuca, Inc.

(2) Related Appeals and Interferences

None.

(3) Status of Claims

Claims 1-47 were previously canceled without prejudice.

Claims 48-77 are pending and stand finally rejected.

(4) Status of Amendments

All amendments have been entered.

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

February 4, 2004

Date of Deposit



Signature

Theresa Popehn

Typed or Printed Name of Person Signing Certificate

(5) Summary of Invention

The invention relates to dietary supplements containing extracts. For example, the present claims recite dietary supplements that contain a grape seed extract, a grape skin extract, and an enzyme, wherein (1) the grape seed extract comprises 3.3 percent or more of the dietary supplement, or (2) the grape seed extract and the grape skin extract combined comprise 32 percent or more of the dietary supplement. The present claims also indicate that the grape seed extract and the grape skin extract contain a flavonoid. In addition, the invention relates to methods for providing a mammal with such dietary supplements. See, e.g., Applicant's specification at page 2, line 26 to page 4, line 5, page 17, lines 16-35, and page 20, line 31 through page 21, line 8.

(6) Issue

Whether claims 48-77 are unpatentable under 35 U.S.C. § 112, first paragraph, for lacking enablement?

(7) Grouping of Claims

Claims 48-77 stand or fall together with respect to the issue of enablement. A different grouping may exist with regard to issues other than this enablement issue.

(8) Argument

Claims 48-77 stand rejected under 35 U.S.C. §112, first paragraph, for lack of enablement on the ground that Applicant's specification does not provide sufficient guidance to enable a person having ordinary skill in the art to make the grape seed extract and grape skin extract recited in the claims. Specifically, the Examiner stated that "there is no teaching in the instant specification what grape seed/skin extract is *actually the invention*," and "[t]he ordinary artisan could *never* determine *what* extract containing *what* flavonoid the claims are referring to, *even after performing undue experimentation* due to lack of information in the [i]nstant specification" (original emphasis).

Applicant respectfully disagrees. Enablement is determined from the viewpoint of "persons of skill in the field of the invention" at the time the patent application was filed. *Ajinomoto Co., Inc. v. Archer DanielsMidland Co.*, 228 F.3d 1338, 56 USPQ2d 1332 (Fed. Cir. 2000). Furthermore, "[a] patent is not a scientific treatise, but a document that presumes a readership skilled in the field of the invention." *Ajinomoto v. Archer DanielsMidland*, citing *W.L. Gore & Associates, Inc. v. Garlock, Inc.* (721 F.2d 1540, 1556, 220 USPQ 303, 315 (Fed. Cir. 1983)).

The presently claimed invention relates to supplements containing extracts. A person skilled in the art of formulating supplements, reading Applicant's specification at the time Applicant filed, certainly would have been able to make and use the claimed dietary supplements without undue experimentation.

Procedures for making extracts, including grape seed extracts and grape skin extracts, were well known for many years prior to Applicant's filing date. This is particularly true given the prior existence of published references that describe routine methods for making grape seed extracts and/or grape skin extracts. For example, U.S. Patent No. 5,484,594 (the '594 patent) discloses multiple methods for making grape seed extracts. In particular, the '594 patent describes making grape seed extracts using standard extraction, concentration, filtration, and ultrafiltration steps. A skilled artisan would have been able to use these standard methods to prepare grape seed extracts.

In addition, the Revilla *et al.* reference (*J. Agric. Food Chem.*, 46:4592-4597 (1998)) compared grape skin extracts produced using various extraction procedures, including those described in Bourzeix *et al.* (*Bull. O.I.V.*, 59:1171-1254 (1986)), Hebrero *et al.* (*Am. J. Enol. Vitic.*, 39:227-233 (1988)), and Cacho *et al.* (*Am. J. Enol. Vitic.*, 43:244-248 (1992)). See, the paragraph extending from the bottom of the left column to the top of the right column on page 4593 of the Revilla *et al.* reference. The fact that multiple references disclosing such extraction procedures were publicly available prior to Applicant's filing date demonstrates that a person skilled in this art would have been well aware of multiple standard methods for making grape seed extracts and grape skin extracts.

The existence of many standard techniques at the time Applicant filed is further supported by the fact that grape seed extracts and grape skin extracts were commercially available from numerous suppliers. These included, for example, Indena (Milan, Italy), Polyphenolics (Canandaigua, NY), InterHealth (Concord, CA), Tri-K Industries (Fanerson, NJ), Freeman Industries (Tuckahoe, NY), Weinstein Nutritional (Irvine, CA), and Brucia Extracts (Shingle Springs, CA). In fact, Applicant's specification teaches that grape seed extracts and grape skin extracts can be obtained from such suppliers. See, Applicant's specification at page 19, lines 2-5 and 14-16.

Dr. Suk Cho, an employee of Melaleuca, Inc. and a co-inventor on U.S. Application Serial No. 09/800,195, confirmed that grape seed extracts and grape skin extracts were well known and commercially available at the time Applicant filed. See, Paragraph 5 of Dr. Cho's Declaration. Dr. Cho also stated that he would have been able to use standard extraction techniques to make grape seed and grape skin extracts. See, Paragraph 6 of Dr. Cho's Declaration.

Moreover, techniques for preparing formulations containing extracts were routine at the time Applicant's specification was filed. A person skilled in the art would have been able to use routine techniques, such as measuring and mixing techniques, to combine a grape seed extract, a grape skin extract, and an enzyme to make the claimed dietary supplements. Indeed, Dr. Cho stated that he would have been able to measure the amounts of grape seed extract, grape skin extract, and enzyme needed to obtain a dietary supplement recited in present claim 48, and would have been able to mix the measured amounts of grape seed extract, grape skin extract, and enzyme to obtain a dietary supplement recited in present claim 48. See, Paragraph 7 of Dr. Cho's Declaration.

In light of the above, Applicant respectfully requests withdrawal of the rejection of claims 48-77 under 35 U.S.C. § 112, first paragraph.

(9) Appendix

An appendix setting forth the claims on appeal is attached.

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The brief fee of \$330 is enclosed. Please apply any other charges or credits to Deposit
Account No. 06-1050.

Respectfully submitted,

Date: February 4, 2004

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Appendix of Claims

48. A dietary supplement comprising a grape seed extract, a grape skin extract, and at least one enzyme, wherein said grape seed extract comprises 3.3 percent or more of said supplement by weight, and wherein said grape seed extract and said grape skin extract contain a flavonoid.
49. The supplement of claim 48, wherein said supplement is in the form of a pill, powder, or tablet.
50. The supplement of claim 48, wherein said at least one enzyme comprises a fungal protease, acid stable protease, or bromelain.
51. The supplement of claim 48, wherein said supplement comprises a bilberry extract.
52. The supplement of claim 48, wherein said supplement comprises a ginkgo biloba extract.
53. The supplement of claim 48, wherein said supplement comprises quercetin.
54. The supplement of claim 48, wherein said at least one enzyme comprises about 24 percent or less of said supplement by weight.
55. The supplement of claim 48, wherein said supplement is PROVEXCV™.
56. The supplement of claim 48, wherein said supplement is PROVEXCV2™.
57. The supplement of claim 48, wherein said supplement comprises a ginkgo biloba extract, a bilberry extract, quercetin, a fungal protease, an acid stable protease, and bromelain.

58. A dietary supplement comprising a grape seed extract, a grape skin extract, and at least one enzyme, wherein said grape seed extract and said grape skin extract combined comprise 32 percent or more of said supplement by weight, and wherein said grape seed extract and said grape skin extract contain a flavonoid.
59. The supplement of claim 58, wherein said supplement is in the form of a pill, powder, or tablet.
60. The supplement of claim 58, wherein said at least one enzyme comprises a fungal protease, acid stable protease, or bromelain.
61. The supplement of claim 58, wherein said supplement comprises a bilberry extract.
62. The supplement of claim 58, wherein said supplement comprises a ginkgo biloba extract.
63. The supplement of claim 58, wherein said supplement comprises quercetin.
64. The supplement of claim 58, wherein said at least one enzyme comprises about 24 percent or less of said supplement by weight.
65. The supplement of claim 58, wherein said supplement is PROVEXCV™.
66. The supplement of claim 58, wherein said supplement is PROVEXCV2™.
67. The supplement of claim 58, wherein said supplement comprises a ginkgo biloba extract, a bilberry extract, quercetin, a fungal protease, an acid stable protease, and bromelain.

68. A method for providing a mammal with a dietary supplement, said method comprising administering said supplement to said mammal, wherein said supplement comprises a grape seed extract, a grape skin extract, and at least one enzyme, wherein said grape seed extract and said grape skin extract contain a flavonoid, and wherein:

(a) said grape seed extract comprises 3.3 percent or more of said supplement by weight,
or

(b) said grape seed extract and said grape skin extract combined comprise 32 percent or more of said supplement by weight.

69. The supplement of claim 68, wherein said supplement is in the form of a pill, powder, or tablet.

70. The supplement of claim 68, wherein said at least one enzyme comprises a fungal protease, acid stable protease, or bromelain.

71. The supplement of claim 68, wherein said supplement comprises a bilberry extract.

72. The supplement of claim 68, wherein said supplement comprises a ginkgo biloba extract.

73. The supplement of claim 68, wherein said supplement comprises quercetin.

74. The supplement of claim 68, wherein said at least one enzyme comprises about 24 percent or less of said supplement by weight.

75. The supplement of claim 68, wherein said supplement is PROVEXCV™.

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76. The supplement of claim 68, wherein said supplement is PROVEXCV2™.
77. The supplement of claim 68, wherein said supplement comprises a ginkgo biloba extract, a bilberry extract, quercetin, a fungal protease, an acid stable protease, and bromelain.

Attorney Docket No. 09143-005001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Lynn Perkes Art Unit : 1614
Serial No. : 09/194,165 Examiner : Patricia Patten
Filed : November 20, 1998
Title : DIETARY SUPPLEMENTS CONTAINING NATURAL INGREDIENTS

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132 OF SUK CHO

I, Suk Cho, declare as follows:

1. I am a citizen of the United States and presently live at 2610 West Charlotte Drive, Idaho Falls, Idaho, 83402.
2. I am presently employed by Melaleuca Inc., and have been so employed since 1998.
3. I received a Ph.D. in organic chemistry from Emory University, Atlanta, Georgia.
4. I have read the above-reference patent application, pending claims, and the Examiner's Office Actions mailed August 13, 2002 and May 1, 2003, including the sections where the Examiner contends that the skilled artisan would not be able to ascertain how to make grape seed extracts and grape skin extracts upon reading the patent application.
5. Grape seed extracts containing flavonoids and grape skin extracts containing flavonoids were well known as of 1997. In fact, many companies sold grape seed and grape skin extracts containing flavonoids as of 1997.
6. As a chemist, I would have been able to use standard extraction techniques to make grape seed and grape skin extracts containing flavonoids as of 1997. For example,

I would have been able to follow standard extraction techniques, such as those described in the references discussed in Applicant's Response filed February 12, 2003 (e.g., U.S. Patent No. 5,484,594), to obtain various grape seed and grape skin extracts containing flavonoids. In general, making flavonoid-containing extracts from grape seeds and grape skins involves using routine, well known procedures.

7. Following the teachings in the above-referenced patent application, I would have been able to make dietary supplements containing grape seed and grape skin extracts containing flavonoids as of 1997. For example, I would have been able to measure the amounts of grape seed extract, grape skin extract, and enzyme needed to obtain a dietary supplement recited in present claim 48. In addition, I would have been able to mix the measured amounts of grape seed extract, grape skin extract, and enzyme to obtain a dietary supplement recited in present claim 48. Measuring and mixing amounts of grape seed extract, grape skin extract, and enzyme involves standard laboratory techniques.
8. Following the teachings in the above-referenced patent application, I would have been able to evaluate dietary supplements for the ability to inhibit platelet aggregation. For example, I would have been able to follow the *ex vivo* procedures described in Example 1 of the above-referenced patent application to determine whether a dietary supplement inhibited platelet aggregation. The techniques used to assess platelet aggregation are standard techniques that can be performed by a trained laboratory technician.
9. Likewise, following the teachings in the above-referenced patent application, I would have been able to evaluate dietary supplements for the ability to protect LDL cholesterol from oxidation. For example, I would have been able to follow the procedures described in Example 2 of the above-referenced patent application to determine whether a dietary supplement protected LDL cholesterol from oxidation. The techniques used to assess oxidation of LDL cholesterol are standard techniques that can be performed by a trained laboratory technician.

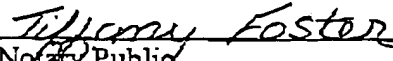
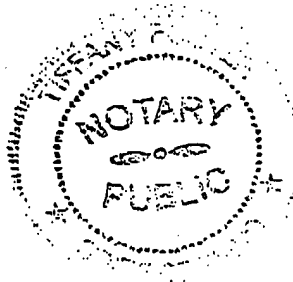
Attorney Docket No. 09143-005001

10. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant patent application or any patent issuing thereon.

Dated: July 1, 2003
Suk Cho

STATE OF Idaho)
) ss.
COUNTY OF Bonniville)

Before me this 1 day of July, 2003, personally appeared Suk Cho known to me to be the person whose name is subscribed to the foregoing Declaration, and acknowledged that he executed the same as his free act and deed for the purposes therein contained.


Tiffany Foster
Notary Public

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Comparison of Several Procedures Used for the Extraction of Anthocyanins from Red Grapes

Eugenio Revilla,* José-María Ryan, and Guillermo Martín-Ortega

Departamento de Química Agrícola, Geología y Geoquímica, Universidad Autónoma de Madrid, 28049 Madrid, Spain

Several procedures for the extraction of grape anthocyanins, based on different methods described in the literature, have been assayed. Results have shown that the use of solvents containing up to 1% of 12 N hydrochloric acid for the extraction of grape anthocyanins resulted in the partial hydrolysis of malvidin 3-*O*-acetylglucoside, leading to important changes in the relative content of anthocyanins in those extracts, despite their higher efficiency in extracting total anthocyanins in some cases. Nevertheless, the use of different neutral solvents resulted in a similar relative content of anthocyanins in the extracts, and some of them could be as efficient in extracting total anthocyanins as other procedures that used an acid solvent for extraction. These data suggest that the use of solvents containing up to 1% of 12 N hydrochloric acid in the extraction of anthocyanins from grapes or from any other plant material containing acetylated anthocyanins should be avoided, especially if the work is related with any type of taxonomic studies.

Keywords: Anthocyanins; acylated anthocyanins; grapes; extraction; HPLC

INTRODUCTION

Anthocyanins are a group of naturally occurring phenolic compounds that are related with the color of several aerial and subterranean organs in many plants (Mazza and Miniatti, 1994). In grapevines (*Vitis vinifera* L.) anthocyanins are accumulated in leaves during senescence and are responsible for the coloration of grape skins in red and rosé cultivars, and in some grape cultivars, named teinturier or redjuice dyer cultivars, these pigments are also accumulated in the grape pulp (Boulton et al., 1995). The accumulation of anthocyanins in grapes begins after the phenological stage known as veraison and is affected by weather conditions, especially light intensity and temperature (Kliewer, 1977), which limit the cultivation of red grape cultivars in regions too cool or too warm during grape maturation. When grapes are harvested, the content of anthocyanins in grapes may be as high as 1000 mg/kg of grapes and even more (Boulton et al., 1995), especially when sugar content in grapes rises to 22%. In red grape cultivars used for wine-making, the amount of anthocyanins present in grapes when harvested may be important in conducting the fermentation and the postfermentation processes in an adequate way to obtain high-quality products. For this reason, several approaches have been taken to understand what changes occur in the content of anthocyanins from veraison to the harvest in several grape cultivars used for wine-making (Piergiorganni and Volonteri, 1983; Darné, 1988; González-San José et al., 1990; Cacho et al., 1992; Fernández-López et al., 1992), showing that the amount of some anthocyanins may decrease in the later stages of the maturation process.

Anthocyanins are unique molecules among plant phenolics because they can be present in plant tissues as different chemical species. At low pH, they are

predominantly present in the form flavylium cation, giving a reddish color in aqueous solutions. At higher pH, the flavylium cation is converted into other species, some of them being uncolored and, under certain conditions, that conversion can be virtually irreversible (Cheminat and Brouillard, 1986). The flavylium cation form is red and stable at a highly acid medium, and this chemical feature of anthocyanins has probably led to a worldwide use of solvents containing mineral or organic acids for the extraction of anthocyanins from plant organs (Ribéreau-Gayon, 1972; Macheix et al., 1990). Nevertheless, Anderson et al. (1970), who identified acetic acid as an acylating agent of anthocyanin pigments in grapes, reported that facile hydrolysis of anthocyanin acetates occurs upon the exposure to trace quantities of mineral acid during the extraction processes, and some authors working on chemotaxonomic studies have reported that the extraction of some acylated anthocyanins under acid conditions may cause their partial or total hydrolysis (Van Sumere et al., 1985; Van Wyck and Winter, 1994). Moreover, Bakker and Timberlake (1985) have shown that artifacts can be obtained by using methanol containing 2% formic acid as the extraction solvent. Despite these reports, several authors have continued to use extraction solvents containing formic acid or hydrochloric acid for studying anthocyanins in grapes (Hebrero et al., 1988; González-San José et al., 1990; Cacho et al., 1992; Fernández-López et al., 1992; Gao and Cahoon, 1995), and some of these methods are also highly time-consuming. Moreover, the use of acid solvents for the extraction of anthocyanins may produce the generation of anthocyanidins from flavanols and proanthocyanidins (Hemingway, 1989), which are present in grape skins to some extent (Bourzeix et al., 1986), and this reaction may result in an overestimation of the total anthocyan content if a colorimetric method is used for this purpose. All of these facts could limit the potential use of those

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Table 1. Some Characteristics of Extraction Procedures Carried out with Skins of Fresh Grapes

| procedure | solvent(s) | vol (mL) | length of extraction (h) | extraction temp (°C) | no. of grapes |
|-----------|--------------------------|---------------|--------------------------|----------------------|---------------|
| 11 | methanol | 60 | 16 | -25 | 100 |
| | 80% methanol | 60 | 4 | 25 | |
| 12 | methanol | 60 | 16 | -25 | 100 |
| | 80% methanol | 60 | 4 | room | |
| | 50% methanol | 60 | 4 | room | |
| | deionized water | 60 | 16 | -25 | |
| | 75% acetone | 60 | 1 | room | |
| 13 | methanol | 60 | 4 | 25 | 100 |
| | methanol | 60 | 12 | 25 | |
| | methanol | 60 | 4 | 25 | |
| 14 | methanol/12 N HCl (99:1) | 60 | 4 | 25 | 100 |
| | methanol/12 N HCl (99:1) | 60 | 12 | 25 | |
| | methanol/12 N HCl (99:1) | 60 | 4 | 25 | |
| | methanol/12 N HCl (99:1) | 60 | 4 | 25 | |
| 15 | methanol/12 N HCl (99:1) | 10 (9 times) | 1 (9 times) | 25 | 50 |
| 16 | methanol | 10 (9 times) | 1 (9 times) | 25 | 50 |
| 17 | methanol/12 N HCl (98:2) | 250 (once) | 24 (once) | 4 | 100 |
| | methanol/12 N HCl (98:2) | 100 | 24 | 4 | |
| | methanol/12 N HCl (98:2) | 100 (4 times) | 24 (4 times) | 4 | |

procedures for routine analysis of anthocyanins in grapevine production.

The aim of our research has been to develop a rapid method, avoiding the use of acid extractions solvents, for the extraction of anthocyanins from grapes that could be used as a routine analysis for enhancing grapevine production. To achieve this purpose, several extraction procedures with different type of solvents (acid or neutral) have been compared using the same set of grapes, and their efficiency in the extraction of anthocyanins has been estimated by colorimetric and chromatographic methods.

EXPERIMENTAL PROCEDURES

Grape Samples. One hundred clusters (~10 kg) of Cabernet Sauvignon grapes were collected in October 1996 from 100 different vines, trained in Guyot double cordon, in a vineyard located in Escalona (province of Toledo, Spain), which has been surveyed by our group for several years. The clusters were collected in plastic bags and were transported to the laboratory at 4 °C. Once there, the grapes were separated from the clusters. Thirty sets of 100 grapes and six sets of 50 grapes were randomly selected and weighed.

Extraction Procedures. Immediately after grape sets were randomly selected and weighed, all of them were processed by carrying out several extraction procedures consisting of two or more sequential steps, the characteristics of which are displayed in Tables 1 and 2. Each extraction procedure has been identified by a two-digit number; the first digit refers to the type of material to be extracted (skins of fresh grapes or entire fresh grapes, designated 1 and 2, respectively), and the second digit refers to the characteristics of the extraction process. Thus, the procedures 11 and 16 involved the extraction of fresh grape skins; and procedures 13 and 23 were carried out under the same conditions, but using skins of fresh grapes and entire fresh grapes, respectively. Some of the procedures used are based on methods for the extraction of phenolic compounds described in the literature. Procedures 12 and 22 are based on the method developed

Table 2. Some Characteristics of Extraction Procedures Carried out with Entire Fresh Grapes

| procedure | solvent(s) | vol (mL) | length of extraction (h) | extraction temp (°C) | no. of grapes |
|-----------|--------------------------|---------------|--------------------------|----------------------|---------------|
| 21 | methanol | 60 | 16 | -25 | 100 |
| | 80% methanol | 60 | 4 | 25 | |
| 22 | methanol | 60 | 16 | -25 | 100 |
| | 80% methanol | 60 | 4 | room | |
| | 50% methanol | 60 | 4 | room | |
| | deionized water | 60 | 16 | -25 | |
| | 75% acetone | 60 | 1 | room | |
| 23 | methanol | 60 | 4 | 25 | 100 |
| | methanol | 60 | 12 | 25 | |
| | methanol | 60 | 4 | 25 | |
| 24 | methanol/12 N HCl (99:1) | 60 | 4 | 25 | 100 |
| | methanol/12 N HCl (99:1) | 60 | 12 | 25 | |
| | methanol/12 N HCl (99:1) | 60 | 4 | 25 | |
| | methanol/12 N HCl (99:1) | 60 | 4 | 25 | |
| 27 | methanol/12 N HCl (98:2) | 250 (once) | 24 (once) | 4 | 100 |
| | methanol/12 N HCl (98:2) | 100 | 24 | 4 | |
| | methanol/12 N HCl (98:2) | 100 (4 times) | 24 (4 times) | 4 | |

by Bourzeix et al. (1986) for extracting phenolic compounds from grape skins and grape seeds; procedures 17 and 27 are based on the method described by Hebrero et al. (1988) for the anthocyanin extraction from grape skins, and procedure 15 is based on the method proposed by Cacho et al. (1992) for extracting anthocyanins from grape skins. As can be noted, each procedure involves two or more successive extractions, and all of them include, as a first step, the immersion of the sample in pure methanol (which contains an adequate proportion of 12 N hydrochloric acid if necessary), followed by grinding in a Kinematica PCU-2 blender for 1 min. When each extraction step was finished, the liquid extract was separated by centrifugation at 3500 rpm and 15 °C for 20 min in a Sorvall RC-5B refrigerated centrifuge, and the residue was submitted to extraction again. For each sample, all of the liquid extracts were combined, and their volume was raised to between 200 and 650 mL with methanol. The extracts were then stored at 4 °C prior to their analysis. Each extraction procedure was carried out in triplicate.

Colorimetric Analysis. Total phenolics were measured in the extracts according to the Folin-Ciocalteu method, following the procedure proposed by Singleton and Rossi (1965), and were expressed as gallic acid equivalents. Total anthocyanins, expressed as malvidin 3-O-glucoside equivalents, were determined using the procedure proposed by Niketic-Aleksic and Hrazdina (1972). All of these measurements were carried out in a Turner 690 UV-vis spectrophotometer.

HPLC Analysis. Several anthocyanins present in the extracts were separated and determined by HPLC. Routine analyses were performed in a chromatograph that consisted of Waters M-510 and M-501 solvent delivery systems, a Waters 680 gradient controller, a Rheodyne 7725 injection valve furnished with a 20 µL loop, a Linear UVIS 200 variable wavelength UV-vis detector set at 520 nm with a sensitivity of 0.01 AUFS, and a Spectra-Physics SP-4290 integrator. In some cases, detection was carried out with a Waters 996 photodiode array detector, and data were acquired and processed in a Waters Millennium workstation, vs 2.15.01, the spectra being registered each second between 250 and 600 nm, with a bandwidth of 1.2 nm. The chromatographic separation was performed on a Waters Nova-Pak C18 cartridge, 150 mm × 3.9 mm, using a Waters Sentry Nova-Pak C18 guard column, 20 mm × 39 mm, both thermostated in a water bath at 32 °C. The mobile phase was a linear gradient of water/acetonitrile (60:40) adjusted at pH 1.2 with perchloric acid (solvent B) in deionized distilled water adjusted at pH 1.2 with perchloric acid (solvent A), at a flow rate of 1.5 mL/min, as it is shown in

Table 3. Linear Gradient Used for the Separation of Anthocyanins by HPLC

| time (min) | solvent A (%) | solvent B (%) | time (min) | solvent A (%) | solvent B (%) |
|------------|---------------|---------------|------------|---------------|---------------|
| 0 | 90 | 10 | 54 | 67 | 33 |
| 5 | 90 | 10 | 55 | 0 | 100 |
| 25 | 80 | 20 | 58 | 0 | 100 |
| 45 | 70 | 30 | 59 | 90 | 10 |

Table 4. Extraction of Total Anthocyanins and Total Phenolics from Skins of Fresh Grapes^a

| extraction procedure | total anthocyanins (mg/100 g of grapes) | total phenolics (mg/100 g of grapes) |
|----------------------|---|--------------------------------------|
| 11 | 79.7 ± 3.7a | 182 ± 10b |
| 12 | 82.8 ± 2.1ab | 248 ± 2c |
| 13 | 88.3 ± 3.1ab | 173 ± 8b |
| 14 | 94.0 ± 7.4b | 243 ± 14c |
| 15 | 91.3 ± 5.6ab | 194 ± 15b |
| 16 | 86.6 ± 4.4ab | 111 ± 3a |
| 17 | 113.9 ± 1.8c | 267 ± 10c |

^aData are mean values for three replications. Values in the same column followed by a common letter are not significantly different ($p < 0.01$).

Table 3. Under these conditions, which are quite close to those described by Bakker and Timberlake (1985), acylated anthocyanins are reasonably stable. The different anthocyanins that have been analyzed were identified on the basis of their retention times and UV-vis spectra by comparison with standards isolated from grapes, as indicated elsewhere. The quantitation of the different substances was carried out in the chromatograms registered at 520 nm by an external standard procedure, using a series of solutions of malvidin 3-*O*-glucoside isolated from grapes. Results are expressed as malvidin 3-*O*-glucoside equivalents.

Statistical Analysis. Statistical analyses of multiple treatment effects were conducted by using one-way analysis of variance with comparison of means made by Duncan's multiple-range test at the 1% level of significance (Duncan, 1957).

RESULTS AND DISCUSSION

Total Phenolics and Total Anthocyanins. Table 4 displays the data corresponding to the levels of total phenolics and total anthocyanins in the different extraction procedures carried out with skins of fresh grapes. As can be noted, the extraction of total phenolics clearly differs in some cases, and two factors seem to play an important role: the total time employed in the extraction and the use of solvents containing hydrochloric acid. Thus, procedure 16, which involves the use of methanol for 9 h, led to the lowest level of extraction of phenolics among the seven procedures used ($p < 0.01$). On the other hand, procedure 17, which used methanol containing 1% of 12 N HCl for a very long time (120 h), led to the highest extraction of phenolics among the seven procedures assayed, but procedures 12 and 14 were as efficient in extracting phenolics as procedure 17 ($p < 0.01$), despite the use of neutral solvents for extraction for the long time (procedure 12) or the relatively short time (20 h) skins were in contact with an acid extraction solvent (procedure 14). The dramatic effect of acidity on the extraction of phenolics is clearly shown by the results corresponding to procedures 15 and 16, which differ only by the presence of hydrochloric acid in the extraction solvent used in procedure 15, and it is a likely consequence of the association of phenolic compounds with cell wall polymers, although the presence of an acid in solvent should increase the rate of extraction of those substances.

Table 5. Extraction of Total Anthocyanins and Total Phenolics from Entire Fresh Grapes^a

| extraction procedure | total anthocyanins (mg/100 g of grapes) | total phenolics (mg/100 g of grapes) |
|----------------------|---|--------------------------------------|
| 21 | 83.5 ± 2.1b | 311 ± 33a |
| 22 | 70.8 ± 1.9a | 430 ± 36b |
| 23 | 86.1 ± 5.5b | 357 ± 7a |
| 24 | 81.1 ± 2.6ab | 510 ± 26c |
| 27 | 110.9 ± 6.4c | 561 ± 7c |

^aData are mean values for three replications. Values in the same column followed by a common letter are not significantly different ($p < 0.01$).

The effects of different factors in the extraction of anthocyanins in experiments carried out with skins of fresh grapes were less evident than they were for total phenolics, but the acidity of solvents used for the extraction and the length of the extraction may play a relevant role in the extraction of those substances. Thus, procedure 17, which used methanol containing 2% of 12 N HCl as solvent, led to a significantly higher extraction of anthocyanins than any other procedure ($p < 0.01$), and procedure 14, which used methanol containing 1% of 12 N HCl as solvent for 20 h, was more efficient ($p < 0.01$) than procedure 11, which used neutral solvents for 20 h. It is very probable that the higher efficiency of procedure 17 was a consequence of the much longer extraction time associated with it compared to others, but it could be hypothesized that the higher efficiency of acid solvents for extracting anthocyanins could partially be explained because flavanols and proanthocyanidins, which are present in grape skins to some extent (Bourzeix et al., 1986), generate anthocyanidins in acid media (Hemingway, 1989). Nevertheless, this hypothesis needs further work to be confirmed.

Table 5 shows the data corresponding to the levels of total phenolics and total anthocyanins in the different experiments carried out with entire fresh grapes. Results obtained for total phenolics were higher than those obtained for assays carried out with skins of fresh grapes (Table 3), as grape seeds contain a very large amount of phenolics (Bourzeix et al., 1986), but the effects of two factors (acidity of solvents and total extraction time) on the extraction of total phenolics from entire fresh grapes were quite similar to those observed in the extraction assays carried out with skins of fresh grapes. It can be seen that the levels of total phenolics were significantly higher ($p < 0.01$) in the extracts corresponding to experiments carried out with acid solvents (procedures 24 and 27) than in the extracts obtained with neutral solvents (procedures 21–23), and this effect should be explained as mentioned above. However, the effects of different factors on the extraction of anthocyanins were less evident than they were for total phenolics: procedure 27, which involved the use of an acid solvent for 120 h, apparently led to the highest extraction ($p < 0.01$) among the five procedures used, but the efficiency of procedures 21, 23, and 24, which lasted 20 h, was similar for extracting anthocyanins ($p < 0.01$), despite the use of neutral solvents (procedures 21 and 23) or of a solvent containing hydrochloric acid (procedure 24). Finally, procedure 22 led to the extraction of a very low amount of anthocyanins. As has been mentioned above, the apparently higher efficiency of procedure 27 for extracting anthocyanins may be a consequence of the much longer extraction time associated with it compared to the others, but the possible

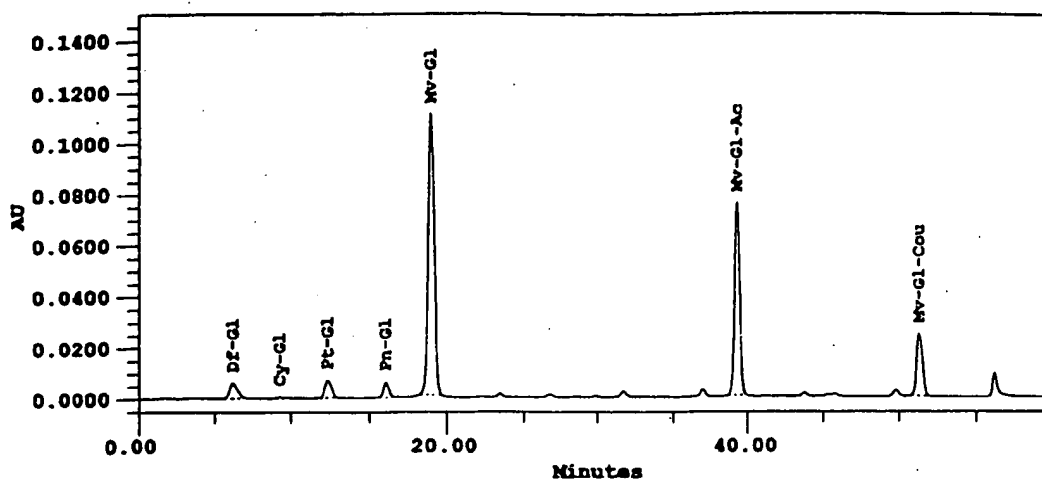


Figure 1. Chromatogram registered at 520 nm corresponding to an extract obtained from skins of fresh grapes, following procedure 11. For key to substances, see text.

Table 6. Extraction of Several Anthocyanins from Skins of Fresh Grapes^a

| | extraction procedure | | | | | | |
|-----------|----------------------|-------------|--------------|--------------|-------------|--------------|-------------|
| | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| Df-Gl | 4.2 ± 0.5a | 4.4 ± 0.4a | 4.9 ± 0.5ab | 5.6 ± 0.5b | 5.8 ± 0.1b | 4.3 ± 0.5a | 5.6 ± 0.5b |
| Cy-Gl | 0.3 ± 0.1a | 0.3 ± 0.1ab | 0.4 ± 0.1abc | 0.5 ± 0.0c | 0.5 ± 0.1bc | 0.4 ± 0.1abc | 0.5 ± 0.1c |
| Pt-Gl | 3.2 ± 0.3a | 3.4 ± 0.2ab | 3.6 ± 0.3abc | 4.4 ± 0.4bcd | 4.6 ± 0.2cd | 3.4 ± 0.4ab | 5.0 ± 0.4d |
| Pn-Gl | 2.2 ± 0.2a | 2.3 ± 0.1a | 2.4 ± 0.3a | 3.2 ± 0.1b | 3.4 ± 0.2bc | 2.3 ± 0.3a | 3.8 ± 0.2c |
| Mv-Gl | 37.9 ± 2.7a | 43.3 ± 2.0a | 42.0 ± 1.2a | 59.2 ± 4.7b | 65.8 ± 2.0b | 41.2 ± 3.8a | 77.8 ± 2.2c |
| Mv-Gl-Ac | 23.1 ± 2.2d | 25.5 ± 0.3e | 25.7 ± 1.1e | 10.2 ± 0.6c | 3.9 ± 0.1b | 25.3 ± 2.1de | 0.5 ± 0.1a |
| Mv-Gl-Cou | 8.1 ± 0.7ab | 9.1 ± 0.2bc | 8.6 ± 0.4ab | 10.1 ± 0.8cd | 10.6 ± 0.1d | 7.4 ± 0.8a | 10.4 ± 0.3d |

^a Results are expressed as mg of Mv-Gl equivalents/100 g of grapes. Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

Table 7. Extraction of Several Anthocyanins from Entire Fresh Grapes^a

| | extraction procedure | | | | |
|-----------|----------------------|-------------|-------------|-------------|-------------|
| | 21 | 22 | 23 | 24 | 27 |
| Df-Gl | 4.2 ± 0.3a | 3.7 ± 0.4a | 3.8 ± 0.4a | 5.1 ± 0.3b | 5.5 ± 0.4b |
| Cy-Gl | 0.3 ± 0.0ab | 0.3 ± 0.1a | 0.3 ± 0.1ab | 0.4 ± 0.1ab | 0.4 ± 0.1b |
| Pt-Gl | 3.2 ± 0.2a | 2.9 ± 0.1a | 3.0 ± 0.3a | 3.7 ± 0.2a | 4.8 ± 0.4b |
| Pn-Gl | 2.1 ± 0.1a | 2.1 ± 0.1a | 2.2 ± 0.1a | 2.5 ± 0.1b | 3.9 ± 0.2c |
| Mv-Gl | 39.6 ± 1.7a | 34.9 ± 1.1a | 39.5 ± 1.7a | 45.3 ± 1.4b | 73.5 ± 3.2c |
| Mv-Gl-Ac | 23.8 ± 1.9cd | 21.8 ± 0.5c | 24.9 ± 0.6d | 13.1 ± 1.2b | 0.3 ± 0.2a |
| Mv-Gl-Cou | 8.6 ± 0.5b | 7.4 ± 0.5a | 8.8 ± 0.3b | 9.2 ± 0.4b | 10.9 ± 0.3c |

^a Results are expressed as mg of Mv-Gl equivalents/100 g of grapes. Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

formation of anthocyanidins from flavanols and proanthocyanidins extracted from grape skins and seeds in an acid media should be considered to a certain extent (Hemingway, 1989).

Extraction of Individual Anthocyanins. Figure 1 shows a chromatogram corresponding to an extract from skins of fresh grapes obtained by procedure 11. Seven different peaks were quantitated and were assigned to delphinidin 3-*O*-glucoside (Df-Gl), cyanidin 3-*O*-glucoside (Cy-Gl), petunidin 3-*O*-glucoside (Pt-Gl), peonidin 3-*O*-glucoside (Pn-Gl), malvidin 3-*O*-glucoside (Mv-Gl), malvidin 3-*O*-acetylglucoside (Mv-Gl-Ac), and malvidin 3-*O*-*p*-coumarylglucoside (Mv-Gl-Cou) on the basis of their chromatographic and spectral characteristics, as has been previously reported (Wulf and Nagel, 1978; Bakker and Timberlake, 1985; Hebrero et al., 1988; Baldi et al., 1995).

Tables 6 and 7 display the data corresponding to the amount of seven anthocyanins determined by HPLC in the extracts obtained using the different procedures assayed for skins of fresh grapes and for entire fresh

grapes, respectively. It can be noticed that the use of solvents which contained hydrochloric acid for extracting anthocyanins apparently led to a higher extraction of nonacylated anthocyanins, and especially malvidin 3-*O*-glucoside, which is the major anthocyanin in Cabernet Sauvignon grapes (Wulf and Nagel, 1978; Darné, 1988). The use of acid solvents (procedures 14, 15, and 17 in the case of experiments with skins of fresh grapes, and procedures 24 and 27 for experiments with entire fresh grapes) led to extracts that contained more malvidin 3-*O*-glucoside than those extracts obtained by procedures that used neutral solvents ($p < 0.01$). The effect was especially intense when methanol containing 2% of 12 N HCl was used as solvent. The other four nonacylated anthocyanins (3-*O*-glucosides of delphinidin, cyanidin, petunidin, and peonidin) behaved similarly, and procedures 17 and 27 led to the highest amount of these molecules in the extracts.

The two acylated anthocyanins that have been analyzed (malvidin 3-*O*-acetylglucose and malvidin 3-*O*-*p*-coumarylglucose) behaved in different ways. Appar-

Table 8. Area Percentages of Seven Anthocyanins Determined by HPLC in Extracts Obtained from Skins of Fresh Grapes^a

| | extraction procedure | | | | | | |
|-----------|----------------------|--------------|--------------|--------------|-------------|-------------|-------------|
| | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| Df-Gl | 5.3 ± 0.3a | 5.0 ± 0.3a | 5.6 ± 0.3abc | 6.0 ± 0.1bc | 6.1 ± 0.0c | 5.1 ± 0.3a | 5.4 ± 0.4ab |
| Cy-Gl | 0.3 ± 0.1a | 0.3 ± 0.1a | 0.5 ± 0.1ab | 0.5 ± 0.0b | 0.5 ± 0.1ab | 0.4 ± 0.0ab | 0.5 ± 0.1ab |
| Pt-Gl | 4.1 ± 0.1a | 3.9 ± 0.1a | 4.1 ± 0.3a | 4.7 ± 0.1b | 4.8 ± 0.0b | 4.1 ± 0.1a | 4.9 ± 0.2b |
| Pn-Gl | 2.8 ± 0.1a | 2.6 ± 0.1a | 2.7 ± 0.3a | 3.5 ± 0.2b | 3.6 ± 0.1b | 2.8 ± 0.1a | 3.7 ± 0.1b |
| Mv-Gl | 48.1 ± 0.6a | 49.0 ± 0.9a | 48.0 ± 0.1a | 63.6 ± 0.3b | 69.7 ± 0.4c | 48.9 ± 0.0a | 75.1 ± 0.7d |
| Mv-Gl-Ac | 29.2 ± 0.5d | 28.9 ± 1.1d | 29.4 ± 0.7d | 10.9 ± 0.2c | 4.1 ± 0.1b | 30.0 ± 0.0d | 0.5 ± 0.1a |
| Mv-Gl-Cou | 10.3 ± 0.2bc | 10.3 ± 0.2bc | 9.8 ± 0.1b | 10.8 ± 0.0cd | 11.2 ± 0.4d | 8.7 ± 0.2a | 10.1 ± 1.2b |

^a Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

Table 9. Area Percentages of Seven Anthocyanins Determined by HPLC in Extracts Obtained from Entire Fresh Grapes^a

| | extraction procedure | | | | | |
|-----------|----------------------|-------------|--------------|-------------|--------------|--|
| | 21 | 22 | 23 | 24 | 27 | |
| Df-Gl | 5.1 ± 0.1ab | 5.0 ± 0.6ab | 4.6 ± 0.3a | 6.4 ± 0.2c | 5.6 ± 0.2b | |
| Cy-Gl | 0.4 ± 0.0a | 0.4 ± 0.1a | 0.4 ± 0.1a | 0.5 ± 0.1a | 0.4 ± 0.0a | |
| Pt-Gl | 4.0 ± 0.2a | 4.0 ± 0.1a | 3.7 ± 0.3a | 4.7 ± 0.2b | 4.9 ± 0.2b | |
| Pn-Gl | 2.6 ± 0.2a | 2.8 ± 0.1ab | 2.7 ± 0.1a | 3.1 ± 0.1b | 3.9 ± 0.1c | |
| Mv-Gl | 48.4 ± 0.8a | 47.9 ± 0.8a | 47.9 ± 0.3a | 57.2 ± 1.1b | 74.0 ± 0.3c | |
| Mv-Gl-Ac | 29.0 ± 0.8c | 29.9 ± 0.2c | 30.2 ± 0.4c | 16.5 ± 0.9b | 0.3 ± 0.2a | |
| Mv-Gl-Cou | 10.5 ± 0.5ab | 10.1 ± 0.4a | 10.6 ± 0.5ab | 11.6 ± 0.2b | 11.0 ± 0.2ab | |

^a Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

ently, the extraction of malvidin 3-*O*-*p*-coumarylglucose followed the same patterns as the extraction of nonacylated anthocyanins. Certainly, the amount of malvidin 3-*O*-*p*-coumarylglucose in extracts obtained from skins of fresh grapes (Table 5) was higher in procedures that involved the use of hydrochloric acid (procedures 14, 15, and 17) than in the others, but differences were not so important as those observed for malvidin 3-*O*-glucoside, and extractions carried out by procedures 12 and 14 were not different ($p < 0.01$). A similar effect was observed for extracts obtained from entire fresh grapes: procedure 27, which involved the use of an acid solvent, led to a more intense extraction of malvidin 3-*O*-*p*-coumarylglucose than procedures 21–23, which used neutral solvents, but the extraction carried out by procedure 24, which involved the use of an acid solvent, was not different ($p < 0.01$) from the extractions carried out by procedures 21 and 22, which used neutral solvents. Consequently, it would be possible to hypothesize that the factors that affect the extraction of malvidin 3-*O*-*p*-coumarylglucose from grapes may be qualitatively different from those that influence the extraction of malvidin 3-*O*-glucoside, except if the high amount of this latter molecule in extracts obtained with acid solvents was the consequence of the hydrolysis of any other acylated derivative. On the other hand, procedures that involved the use of acidic solvents (procedures 14, 15, 17, 24, and 27) led to the extraction of a lower amount of malvidin 3-*O*-acetylglucose ($p < 0.01$) than procedures that used neutral solvents. The effect of using methanol containing 2% of 12 N HCl as solvent was especially dramatic, and the extracts obtained with this solvent contained a very low amount of malvidin 3-*O*-acetylglucose. The effect of other factors on the extraction of nonacylated anthocyanins and their acetyl derivatives, such as total extraction time, extraction temperature, or the number of successive extractions, seem to be less important than the use of acidic solvents for their extraction.

The results obtained suggest that the presence of hydrochloric acid in the extraction solvent originates the partial hydrolysis of acetylated anthocyanins, as it was reported previously by Anderson et al. (1970), Van Sumere et al. (1985), and Van Wyk and Winter (1994). Data on the relative content of the seven anthocyanins analyzed by HPLC in the extracts obtained in the different experiments, expressed as area percentages and summarized in Tables 8 and 9, would make this question clear. The relative contents of malvidin 3-*O*-glucoside were quite similar for all of the extracts obtained with neutral solvents (procedures 11–13, 16, and 21–23), ranging from 47.9% (procedure 22) to 49.0% (procedure 12), and, in the same way, the relative contents of malvidin 3-*O*-acetylglucose were quite similar for all of the extractions carried out with neutral solvents, ranging from 28.9% (procedure 12) to 30.2% (procedure 23). The use of acid solvents increased the relative content of malvidin 3-*O*-glucoside in the extracts ($p < 0.01$), which may be as high as 75.1% (procedure 17), and consequently reduced the relative content of malvidin 3-*O*-acetylglucose ($p < 0.01$), which may be as low as 0.3% (procedure 27).

Furthermore, the relative contents of the other four nonacylated anthocyanins (the 3-*O*-glucosides of delphinidin, cyanidin, petunidin, and peonidin) were higher in the extracts obtained by procedures that involved the use of acid solvents than in those obtained when neutral solvents were used, probably as a consequence of the hydrolysis of their acetylated derivatives, because the apparent extraction of those nonacylated anthocyanins was more intense when acid solvents were used (Tables 6 and 7). Unfortunately, it was not possible to evaluate the amount of the acetyl derivatives of those anthocyanins present in the extracts.

These data point out that the use of solvents containing up to 1% of 12 N hydrochloric acid produces the partial hydrolysis of some acetylated anthocyanins during extraction. As a consequence, the use of experi-

mental protocols for extracting anthocyanins using solvents that contained up to 1% of 12 N hydrochloric acid from grapes or from any other plant material containing acetylated anthocyanins should be avoided, especially if the work is related with any type of taxonomic studies. Any extraction protocol that uses other mineral and organic acids, and even solvents containing hydrochloric acid at lower concentrations than those used in our experiments, should be tested against an extraction procedure that involves the use of neutral solvents, to avoid partial hydrolysis of acetylated anthocyanins or even the formation of laboratory artifacts, as those mentioned by Bakker and Timberlake (1985) when formic acid is used for the extraction of anthocyanins. The relatively high amount of malvidin 3-O-acetylglucoside present in Cabernet Sauvignon grapes makes them good material for testing extraction solvents intended to be used for anthocyanin analysis.

Some of the procedures used, and especially procedure 23, could give information of interest about the content of anthocyanins in grapes shortly after sampling (~24 h) and even complementary information about the global phenolic status of grapes. These data could be useful for the better conducting of red wine fermentation, as anthocyanins and other phenolic compounds play a very relevant role in the characteristics of red wines.

LITERATURE CITED

- Anderson, D. W.; Gueffroy, D. E.; Webb, A. D.; Kepner, R. E. Identification of acetic acid as an acylating agent of anthocyanin pigment in grapes. *Phytochemistry* 1970, 9, 1579–1583.
- Bakker, J.; Timberlake, C. F. The distribution of anthocyanins in grape skin extracts of Port wine cultivars as determined by high performance liquid chromatography. *J. Sci. Food Agric.* 1985, 36, 1315–1324.
- Baldi A.; Romani, A.; Mullinacci, N.; Vincieri, F. F.; Casetta, B. HPLC/MS application to anthocyanins of *Vitis vinifera* L. *J. Agric. Food Chem.* 1995, 43, 2104–2109.
- Boulton, R. B.; Singleton, V. L.; Bisson, L. F.; Kunkee, R. E. *Principles and Practices of Winemaking*; Chapman & Hall: New York, 1995.
- Bourzeix, M.; Weyland, D.; Heredia, N. Étude des catéchuins et des procyanidols de la grappe de raisin, du vin, et d'autres dérivés de la vigne (A study of catechins and procyanidins of grape clusters, the wine, and other byproducts of the vine). *Bull. O.I.V.* 1986, 59, 1171–1254.
- Cacho, J.; Fernández, P.; Ferreira, V.; Castell, J. E. Evolution of five anthocyanidin-3-glucosides in the skin of Tempranillo, Moristel, and Garnacha grape varieties and influence of climatological variables. *Am. J. Enol. Vitic.* 1992, 43, 244–248.
- Cheminat, A.; Brouillard, R. PMR investigation of 3-O-(β -D-glucosyl)-malvidin structural transformations in aqueous solutions. *Tetrahedron Lett.* 1986, 27, 4457–4460.
- Darné, G. Evolution des différentes anthocyanes des pellicules de Cabernet Sauvignon au cours du développement des baies (Evolution of different anthocyanins in the skins of Cabernet Sauvignon during grape development). *Connaiss. Vigne Vin* 1988, 22, 225–236.
- Duncan, D. B. Multiple range tests for correlated and heteroscedastic means. *Biometrics* 1957, 13, 164–176.
- Fernández-López, J. A.; Hidalgo, V.; Almela, L.; López-Roca, J. M. Quantitative changes in anthocyanin pigments of *Vitis vinifera* cv. Monastrell during maturation. *J. Sci. Food Agric.* 1992, 58, 153–155.
- Gao, Y.; Cahoon, G. A. High performance liquid chromatographic analysis of anthocyanins in the red seedless table grape Reliance. *Am. J. Enol. Vitic.* 1995, 46, 339–345.
- González-San José, M. L.; Barrón, L. J. R.; Díez, C. Evolution of anthocyanins during maturation of Tempranillo grape variety (*Vitis vinifera*) using polynomial regression models. *J. Sci. Food Agric.* 1990, 51, 337–343.
- Hebrero, E.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. High performance liquid chromatography-diode array spectroscopy identification of anthocyanins of *Vitis vinifera* variety Tempranillo. *Am. J. Enol. Vitic.* 1988, 39, 227–233.
- Hemingway, R. W. Reactions at the interflavonoid bond of proanthocyanins. In *Chemistry and Significance of Condensed Tannins*; Hemingway, R. H., Karchesy, J. J., Eds.; Plenum Press: New York, 1989.
- Klewer, W. M. Influence of temperature, solar radiation and nitrogen on coloration and composition of Emperor grapes. *Am. J. Enol. Vitic.* 1977, 28, 96–103.
- Macheix, J. J.; Fleuriet, A.; Billot, J. *Fruit Phenolics*; CRC Press: Boca Raton, FL, 1990.
- Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables and Grains*; CRC Press: Boca Raton, FL, 1993.
- Niketic-Aleksic, G. K.; Hrazdina, G. Quantitative analysis of the anthocyanin content in grape juices and wines. *Lebensm. Wiss. Technol.* 1972, 5, 163–165.
- Piergovanni, L.; Volonterio, G. Studio della frazione antocianica delle uve. Nota II. Variazione di composizione durante la maturazione (A study of the anthocyanins fraction of grapes. II. Changes in its composition during ripening). *Tecnol. Aliment. Imbottigliamento* 1983, 6, 22–28.
- Ribéreau-Gayon, P. *Plant Phenolics*; Oliver and Boyd: Edinburgh, 1972.
- Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent. *Am. J. Enol. Vitic.* 1965, 16, 144–158.
- Van Sumere, C. F.; Vande Castele, K.; De Loose, R.; Heursel, J. Reversed phase-HPLC analysis of flavonoids and the biochemical identification of cultivars of evergreen Azalea. In *Annual Proceedings of the Phytochemical Society of Europe*; Van Sumere, C. F., Lea, P. J., Eds.; Clarendon Press: Oxford, U.K., 1985; Vol. 25.
- Van Wyk, B. E.; Winter, P. J. D. Chemotaxonomic value of anthocyanins in *Podalyria* and *Virgilia* (Tribe Podalyrieae: Fabaceae). *Biochem. Syst. Ecol.* 1994, 22, 813–818.
- Wulf, L. W.; Nagel, C. W. High-pressure liquid chromatographic separation of anthocyanins of *Vitis vinifera*. *Am. J. Enol. Vitic.* 1976, 29, 42–49.

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